ISOLATION AND CHARACTERIZATION OF GENES INDUCED IN *ASPERGILLUS NIDULANS* AFTER TRANSFER TO PECTIN AND GLUCOSE-STARVED CONDITIONS

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CHAPTER 1

INTRODUCTION

The fungi are important in turnover of biomass and other materials, and some of them cause diseases in plants and animals, including humans, through direct attack and/or through toxin secretion. Many fungi have the ability to synthesize compounds, which have significant value in the food, drug, and chemical industries. Fungi that are either parasitic on plants or saprophytic produce a wide range of plant cell wall degrading enzymes.

Aspergillus nidulans is one such saprophytic filamentous fungus, which survives on dead and decaying organic matter (Prade et al., 1999). The advantages of using *A. nidulans* in the lab include rapid growth on defined media, compact colony morphology, asexual spores, meiotic genetics (sexual) and mitotic analysis (parasexual). Besides, the genome has been recently sequenced and the sequence data has been released by the Whitehead Institute of Genomics Research (http://www.broad.mit.edu/annotation/fungi/aspergillus/). The relative ease of handling *A. nidulans* in the lab facilitates the investigation of genetics and molecular biology of this fungus and has made this organism a model one to study.

The most abundant materials encountered by the fungus, *A. nidulans* while surviving on decayed organic matter are plant cell walls, which are rich in pectin, cellulose, and hemicellulose. Apart from providing mechanical strength, plant cell walls serve as an extracellular pathway for the continuous movement of water and ions within the plant and provide protection against invaders.

Primary plant cell walls are a complex assembly of polysaccharides, which include cellulose, hemicellulose, pectins and proteins with small quantities of phenols. Secondary cell walls are heavily thickened; contain almost no pectin, less protein and almost 30% of lignin by weight (McNeil et al., 1984).

Cellulose, the main carbohydrate made by plants is a linear polymer of glucose subunits connected by β-(1->4) glycosidic linkages (Beguin P 1990, Beguin et al., 1994). Cellulolytic microorganisms breakdown cellulose to glucose. Induction of the cellulase genes is brought about by cellulose, its derivatives, and sophorose (Saloheimo et al., 2000). Repression in the presence of glucose is brought about by CreA protein that negatively regulates genes encoding cellulases, hemicellulases and pectinases (Strauss et al., 1995, Margolles-Clark et al., 1997, Ruijter et al., 1997). CreA is a DNA binding protein that binds the target consensus sequence 5'-SYGGRG-3' through a pair of C_2H_2 zinc fingers which show 84% identity at the amino acid level with the DNA binding region of the Mig1 protein of *Saccharomyces cerevisae* (Drysdale et al., 1993, Orejas et al., 1999).

Hemicellulose is composed of complex carbohydrates like xyloglucan (XG) in primary walls and xylans (XY) in secondary walls. Endoxylanases attack xylan at the D-xylose backbone and reduces the degree of polymerization which facilitates uptake by cells (Thompson JA 1993). The transcriptional activator, XlnR regulates the expression of genes coding for the main xylanolytic enzymes as well as genes involved in cellulose degradation (Van Peij et al., 1998).

Pectins constitute about 40% of the primary plant cell wall. There are two types of backbones in pectin that are most likely covalently linked (Ridley et al., 2001, Prade et al., 1999). They are: homogalacturonan (HG) and rhamnogalacturonan (RG). HG consists of

long stretches of 1,4 linked α-D-galactopyranosyluronic acid residues, some of which are methyl esterified (Thibault et al., 1993). The other backbone, RG, contains galacturonic acid and rhamnose (GalA-Rha) dimer repeats with variable side chains containing arabinose and galactose. Enzymes that degrade RG side chains are: arabinases, α-L arabinofuranosidase, and galactanases (Ruijter et al., 1997, Yamaguchi et al., 1995).

Fungi and bacteria are highly effective in hydrolyzing pectin into metabolizable energy sources. Currently there is little information about what controls the level of gene expression of many cell wall degrading enzymes and the extent to which the central metabolism changes when fungi are given cell wall polysaccharides like pectin as their source of food.

Previous studies demonstrated that *A. nidulans* produces a wide range of enzymes when grown on a mixture of complex plant cell wall polysaccharides and a limited number of enzymes when grown on a single polymer (Mort and Prade, unpublished results). In order to understand the regulation of genes encoding enzymes required to degrade complex carbohydrates, it was essential to isolate the genes involved in the process. The studies conducted here using Negative Subtraction Hybridization as described in Chapter 2 helped us to isolate the cDNAs of messages induced during fungal growth on complex carbohydrates. Microarray studies conducted with the isolated cDNAs revealed an interesting pattern as discussed in Chapter 3, thus giving a snapshot of the fungal metabolism at different times and growth conditions. Chapter 2 in this thesis has been published in 2004 in BMC Genomics and Chapter 3 has already been submitted.

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CHAPTER 2

NEGATIVE SUBTRACTION HYBRIDIZATION: AN EFFICIENT METHOD TO ISOLATE LARGE NUMBERS OF CONDITION-SPECIFIC CDNAS

Introduction

High throughput analysis of differentially expressed genes has been widely used to address a multitude of biological questions. For such analysis, a large collection of cDNA molecules representing the potential genes of interest is useful. A variety of techniques have been used to identify the cDNAs representing genes of interest associated with various biological processes. Some of the techniques include characterization of expressed sequence tags (EST) (Adams et al., 1991), suppressive subtractive hybridization (SSH) (Diatchenko et al., 1996, 1999), and representational difference analysis (RDA) (Lisitysn et al., 1993).

EST sequencing first became popular in 1991 (Adams et al., 1991). The basic scheme of an EST sequencing project relies on a cDNA library constructed from a tissue of interest under a particular condition from which randomly isolated clones are sequenced until further sequencing no longer yields an acceptable frequency of identifying novel cDNAs. A drawback faced in this method is the repeated sequencing of abundant transcripts and, hence, the expense and effort of sequencing them.

The abundance of mRNAs in a typical eukaryotic cell can be divided into rare transcripts present at 1-15 copies per cell, moderately abundant transcripts present at 100-500 copies per cell, and abundant transcripts present at over 1,000 copies per cell (Soares et al.,

1994). The identification of rare mRNAs from a cDNA library, based on a random selection scheme, can be difficult because of their low representation. Construction of normalized cDNA libraries based on reassociation kinetics has been used to significantly reduce the representation of abundant transcripts, thereby increasing the chances of obtaining the rare cDNAs (Soares et al., 1994). However, normalization often results in a bias towards small inserts (Carninci et al., 2000). Widely used techniques such as SSH can be useful for identifying gene expression differences at the mRNA level, but do not adequately address the problem of redundant transcripts (Hedrick et al., 1984).

Another technique called RDA has been used to clone differentially expressed cDNAs (Welford et al., 1998). However, this technique does not solve the problem of isolating rare mRNAs from the population of abundant mRNA species (Hubank et al., 1994). There are other methods such as mRNA differential display and RNA fingerprinting by arbitrary primed PCR but both of these methods are unsuitable for experiments where the expression levels of relatively few of the genes are expected to vary (Liang et al., 1992, Welsh et al., 1992, Bauer et al., 1994, Malhotra et al., 1998). In addition, the sensitivity of these methods depends on the primer sequences, the concentration of the template, and its potential binding sites, and sometimes both of the above methods report a high number of false positives (Wan et al., 1996).

Here we report a simple and very effective method to isolate cDNAs for transcripts induced by changes in growth conditions or differentiation. For this study we were interested in isolating cDNAs induced by switching a fungus from growth on glucose to growth on selected polysaccharides. Approximately 4,700 contigs from 12,320 ESTs were already available from a cDNA library representing transcripts isolated from glucose-grown *A.*

nidulans during asexual development (Prade et al., 2001). Our goals were to expand the cDNA collection without repeated sequencing of previously identified ESTs and to find as many cDNAs as possible that are specifically induced in complex polysaccharide metabolism.

Materials and Methods

Isolation of RNA and construction of cDNA library

The *A. nidulans* strain FGSC C26 (genotype: *biA1 veA1*) was inoculated at 10^6 spores per ml of minimal medium containing 1% glucose and grown at 37°C for 18 hr with constant shaking at 300 rpm. Minimal medium with the appropriate supplements was prepared as described by Pontecorvo (Pontecarvo G., 1969). Fungal mycelia present in 100 ml of the culture were collected, washed with water and added to different 250 ml flasks containing minimal medium supplemented with 1% of a single complex carbon source representing those found in plant polysaccharides. The fungal cultures were then grown at 37°C, pH 6.5 at 200 rpm for an additional 8 hr in minimal medium supplemented with one of the following carbon sources: carboxy methyl cellulose (Sigma), xyloglucan (Megazyme), rhamnogalacturonan (Megazyme), pectin (Sigma), karaya gum (Sigma), gum arabic (Sigma), locust bean gum (Sigma), arabinogalactan protein (provided by Dr. Jinhua An, Pharmagenesis), arabinoxylan (Megazyme), pectic galactan (Megazyme), xylan (Sigma) or arabinan (Megazyme). Total RNA from lyophilized mycelia of *A. nidulans* was isolated using Tri reagent (Life Technologies) based on the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski P., 1993). Equal amounts of the total RNA from cultures grown in each medium were combined to create a single pool of total RNA. A

non-normalized cDNA library from the pooled RNA from *A. nidulans* was constructed by Life Technologies, Inc. using the vector, pCMVSport 6.0. The titer of the non-normalized library was 5.06 X 10^6 cfu/ml, and 98% of the clones from the library had inserts with the average insert size being 1.49 kb. The plasmid library was plated on 150 mm petridishes containing LB-Amp (100 µg/ml) such that each 150 mm plate had approximately 1,000 colonies.

Screening of cDNA library and negative subtraction hybridization

Probes used for screening the cDNA library were made from cDNA reverse transcribed from total RNA of *A. nidulans* grown in Minimal Medium containing 1% glucose at 37°C for 18 hr, collected, washed and transferred to fresh medium of the same composition and grown for an additional 8 hr. The cDNAs were PCR amplified using Advantage ® cDNA PCR Kit according to the manufacturer's instructions and labeled with the Digoxygenin (DIG) labeling and detection system from Roche Molecular Biochemicals using the random prime labeling method according to the manufacturer's instructions. DIG-labeled cDNAs used as probes were adjusted to a final concentration of 20 ng of probe/ml of hybridization buffer. The labeled probes were used to screen the cDNA library of *A. nidulans* by colony hybridization (Sambrook et al., 1989).

Stringency washes following hybridization were performed according to the manufacturer's instructions supplied by Roche Molecular Biochemicals. The membranes were exposed to the chemiluminescent substrate CDP-Star between two plastic sheets, allowed to incubate for 5 min, sealed in plastic bags and then exposed to Lumi-film (Roche Molecular Biochemicals) for detection. Exposure times of 5 min, 10 min and 20 min were

taken. The X-Ray films were aligned to the nylon membranes attached in the autoradiography cassettes, then aligned to the LB-Amp plates containing the colonies and the positively hybridized colonies were flagged. Those colonies which did not hybridize to the probe (cDNAs from glucose-grown fungus) were selected and grown in 200 µl of LB-Amp (100 µg/ml) in a 96-well plate at 37°C for 14-16 hr.

Cultures from the 96-well plates were gridded on nylon membranes (Amersham Pharmacia Biotech) (Buitkamp et al., 2000) for secondary hybridization using the same probe as used in the primary hybridization. Glycerol stocks of the clones selected at the primary hybridization stage were also maintained.

The clones which were negative after the second round of hybridization were selected and 100 µl of their glycerol stocks were added to 1000 µl of Terrific Broth (Amp, 100 µg/ml) in 96-well culture blocks and grown at 37°C for 16 hr with shaking at 200 rpm. Plasmid DNAs were prepared using the 96-well alkaline lysis miniprep kit from Edge Biosystems according to the manufacturer's instructions. The DNAs were PCR amplified in 96-well thin-walled V-bottom microtitre plates (USA Scientific) using DyeDeoxy "Terminator PRISM" mix using the following program: 96°C for 30 s, 45°C for 15 s, 60°C for 4 min for 49 cycles (Gong et al., 2001). The PCR products were purified to remove unincorporated dyes and primers using the 96-well gel filtration and purification kit from Edge Biosystems and sequenced on an ABI 3700 sequencer.

The raw sequences (ABI chromatograms) were processed using PipeOnline (http://bioinfo.okstate.edu/pipeonline) (Ayoubi et al., 2002) for functional annotation. PipeOnline (POL) is a fully automated EST processing program designed to take raw

sequence trace files as input, call bases, remove vector sequences, assemble contigs and annotate function to them wherever possible.

Testing of the negatives using microarray experiments

Inserts from the clones from our inventory of negatives were PCR amplified using T7 and SP6 primers. Thermal cycling conditions consisted of an initial denaturation of 96^{o}C for 3 min, followed by 35 cycles of 94^{o}C for 30 s, 45^{o}C for 45 s, 72^{o}C for 1 min 30 s with a final extension at 72^{o}C for 10 min. The quality of the PCR products was examined by electrophoresis by using 5 µl of the products on 1% agarose gel where 98% of the PCR products revealed a single band of 500 bp or longer and were chosen for microarray analysis. The unpurified PCR products (Diehl et al., 2002) were resuspended in Micro Spotting Plus Solution (Telechem). They were printed at a final concentration of 250 ng/µl on amino-silane coated slides (Corning Cat # 40005) in quadruplicate at room temperature and 50% relative humidity using a PixSys 5500 microarrayer (Genomic Solutions) fitted with Majer Precision Pins. Various heterologous external controls, control sets from Ambion, as well as different negative (empty vectors) and internal positive controls including pooled cDNAs which were PCR amplified from the non-subtracted cDNA library were used in the arrays. After printing, each slide was rehydrated by holding the slide with the array side down over a beaker of steaming water for 1 s and snap-dried on a warm hot plate. The slides were baked at 85^{o}C for 3 hr followed by UV cross-linking using a Stratalinker. Prior to hybridization with labeled cDNAs, the features immobilized on the slides were denatured by immersing the slides in boiling water for 2 min followed by snap-cooling on a bed of ice. The slides were

dried by brief centrifugation followed by prehybridization at 42^{o}C in 0.5% SDS, 0.01% BSA, 6 X SSC and 25% formamide for 1 hr and washed with nanopure water at room temperature.

Preparation of labeled cDNAs and the hybridization procedure

Total RNA was isolated from *A. nidulans* grown in different complex carbohydrates as described before, and 5 µl of RNA isolated from tissues grown in each condition was checked in a denaturing formaldehyde/agarose gel. RNA samples with an A_{260}/A_{280} ratio between 1.8 and 2.0 and sharp ribosomal RNA bands were selected for use in preparing labeled cDNAs for hybridization. Twenty-five micrograms of total RNA was used for labeling using Genisphere's Array 350 hybridization kit according to the manufacturer's instructions. Each hybridization for a particular condition tested was repeated three times for statistical validation. Labeled cDNAs for the replicate hybridizations were prepared from three independent fungal cultures. They were incubated at 80^{o}C for 10 min followed by 42^{o}C for 10 min before applying to the prewarmed and prehybridized array. The formamide based hybridization buffer (provided with the kit) used for the experiments enabled us to perform the hybridization at 42^{o}C. A 22 X 40 mm cover slip (Grace Bio-Lab, Bend, OR) was carefully placed on the slide taking care not to create any bubbles, and the slides were incubated overnight in a CMT-Hybridization chamber (Corning Inc., Corning, NY). The humid atmosphere inside the chamber was maintained by applying 15 µl of 3 X SSC in the reservoir wells. The hybridization and the stringency washes were also done according to the manufacturer's instructions. The arrays were scanned using Scan Array Express from Perkin-Elmer.

Image extraction and data analysis

Scanned images were analyzed using the software package, Gene Pix Pro 4.0 (Axon Inc.). Spots with signal intensities lower than background, scratched spots and spots covered with dust were flagged and excluded from further analysis. Local background was subtracted from the signal intensity of each spot on the array. For between-slide normalization, a normalization factor was calculated from the mean of the background subtracted median pixel intensities of the *A. nidulans* pooled non-subtracted cDNAs which were used as internal controls. This normalization factor was then used in Gen Pix Pro 4.0 to normalize the feature signal intensities in each slide. Normalized signals from replicate spots within each treatment were averaged, and the values were used to determine the detection of cDNAs under particular conditions. Detection of cDNAs was done by comparing the normalized signal intensities of each spot to the mean of the background subtracted median pixel intensities of the negative controls. Visualization of the intensities of cDNAs across the wide spectrum of inducing substances was done by using GENESIS software (Sturn et al., 2002). All the array results are deposited at NCBI Gene Expression Omnibus under the platform accession number GPL566 and the series number GSE783 (http://www.ncbi.nlm.nih.gov/geo).

Northern Analysis

Northern blots were prepared following standard methods (Sambrook et al., 1989) using 10 µg of *A. nidulans* total RNA per lane. The cDNAs used as probes were selected from our collection of negative clones, digested with Eco RI and Hind III, separated by electrophoresis on a 1% agarose gel, gel-extracted (Qiagen Gel Extraction Kit) and labeled

with DIG random prime labeling system from Roche Molecular Biochemicals according to the manufacturer's instructions. The membranes were hybridized with the labeled probe in DIG Easy Hyb at a final concentration of 20 ng/ml according to the manufacturer's instructions. Before re-use, each blot was stripped twice in 100 ml of boiling water containing 0.1% SDS and shaken on a rocking platform for 10 min, and washed twice in 1 X wash buffer (100mM maleic acid pH 7.5; 150mM NaCl; 0.3 % (v/v) Tween 20) for 10 min.

Results

Isolation, sequencing, and classification of NSH-derived ESTs

A non-normalized, non-amplified cDNA plasmid library was constructed from pooled RNAs extracted from *A. nidulans* grown on different polysaccharides (see materials and methods). If a fungal culture is given a mixture of complex polysaccharides the cells will most likely utilize one class of polysaccharide preferentially over another (Mort et al., unpublished results). Keeping this in mind, we grew fungal cultures individually in twelve different polysaccharides. RNA was extracted from each culture and pooled to make a composite cDNA library. We hoped these twelve different polysaccharides would represent all of the sugars and sugar linkages present in plant cell walls and would thereby induce a wide variety of enzymes necessary for the degradation and metabolism of the complex carbohydrates.

About 100,000 colonies from the cDNA library were screened with DIG-labeled cDNAs reverse transcribed from RNAs extracted from glucose-grown fungus. Colonies showing no or very faint hybridization signals (negatives), indicating potentially condition-specific transcripts, were selected and transferred to 96-well plates, and then subjected to

secondary hybridization using the same probe. Out of a total of approximately 100,000 colonies plated, 8,000 negatives were counted and then finally 3532 (3.5% of those screened) well isolated colonies were selected as negatives for further analysis. Plasmid DNAs were isolated, the plasmid inserts were end-sequenced and the sequence data were processed using PipeOnline (POL) (Ayoubi et al., 2002), which generated a database containing 2,039 unique contigs assembled from overlapping sequences. All NSH derived EST sequences were deposited at NCBI's dbEST database (http://www.ncbi.nlm.nih.gov/dbEST) with accession numbers ranging from CK445320 to CK449149 and from CK468506 to CK468532.

Figure 1 shows the distribution of predicted functions of the proteins represented by the ESTs as classified by POL using the best BLASTX hit with an expectation value E ≤ 1e-3. The ESTs were grouped as follows: 1% into cell structure, 4% into cell wall enzymes, 4% into electron transport, 4% into signal transduction, 5% into membrane transport, 9% into information pathways, 21% into metabolism and bioenergetics, 37% were unclassified and 15% were unknown. Unclassified ESTs are those sequences that had a BLASTX hit with an expectation value E ≤ 1e-3, but no "known" function attributed to them. ESTs with no significant similarity to known peptides were designated "unknown". From Figure 1 it is evident that genes of unclassified and unknown function gave rise to half of the ESTs found in the NSH screen.

Figure 2 shows the percentage of contigs containing a given number of NSH-ESTs plotted against the number of ESTs per contig. The majority (75%) of contigs contain one NSH-EST, which indicates low sequence redundancy in the NSH collection and the sampling from our library may also have been far from being complete.

Validation of the negatives using slide-based hybridization

If the NSH method worked as predicted, one would expect most clones selected to contain cDNA inserts from genes not expressed in glucose-grown fungus but induced by forcing the fungus to utilize a complex carbohydrate as its carbon source or de-repressed after removal of glucose. To test this, clones representing about half of the contigs from our inventory of negatives which were also not present in the previous EST collection were PCR amplified and microarrayed on glass slides. A subset of the polysaccharides used for library construction namely pectin, arabinan, carboxymethyl cellulose, locust bean gum, and gum arabic were used separately as substrates for fungal cultures, and from those individual cultures labeled cDNAs were prepared. Cultures grown on glucose and incubated in the absence of glucose (starved) were also used to prepare cDNAs. The cDNAs prepared from each condition were used in single channel non-competitive slide-based hybridizations.

For each carbohydrate tested, background-subtracted hybridization signals were normalized between slides based on the intensity of the controls and ranked from 0-3 depending on the normalized signal intensity. Figure 3 summarizes all of the results by representing the average relative fluorescence intensity of each gene in each treatment by one of three shades of red or by black if it was deemed non-detectable. The order of the features appearing in the figure is relative to the intensity of signal obtained from glucose grown labeled fungal cDNA (right-hand column) ranked from the highest to the lowest and secondarily by the sum of relative fluorescence intensity levels in all the other hybridizations. The features can be grouped into four categories: 1) Those that appear to be expressed at high levels in glucose and at high or lower levels in starvation or complex carbohydrates 2) Those

that are expressed to a low extent in glucose-grown cultures but are expressed to higher levels when grown in other conditions tested 3) Those that are non-detectable in the glucose-grown cultures but are expressed in one or more of the other conditions 4) Those that are apparently not expressed under any condition tested

These results also revealed substrate-specific expression of genes. For instance, cDNA encoding exopolygalacturonase hybridized only to labeled cDNA made from pectin-grown fungal mycelium. Similarly, cDNA encoding endo-arabinanase hybridized only to labeled cDNA made from arabinan and arabinogalactan protein-grown fungal cultures but not to labeled cDNA extracted from fungal cultures grown in glucose (Figure 3).

The intensity of hybridization to several cDNAs for which there was a relatively high redundancy in our NSH collection were investigated to determine whether they were actually highly expressed in fungal cultures grown in any of the conditions other than glucose. A cDNA encoding phosphoenol pyruvate carboxykinase (PEPCK), involved in gluconeogenesis, (picked 9 times during the NSH screen) hybridized at very high levels to labeled cDNA extracted from starved cultures but only weakly to labeled cDNA prepared from glucose-grown fungal cultures.

Twenty-one clones selected during the NSH procedure contained inserts with sequences matching that of a 30 kDa heat shock protein. The corresponding cDNA hybridized very strongly to labeled cDNAs from fungus grown on all carbon sources, including glucose. Another cDNA, sequenced 14 times and with an unknown function, hybridized to a lesser extent to labeled cDNA from glucose-grown fungus compared to that of fungus grown on all of the complex polysaccharides or incubated in no carbon source. The cDNA encoding cell wall galactomannoprotein (sequenced 6 times in the NSH

collection) gave no signal from glucose-grown fungus, but a low signal in starvation and three of the complex carbohydrates. There were also 80 features on the microarray which did not hybridize to any of the labeled cDNAs tested.

Northern evaluation of the negative subtraction technique and microarray data

Several of the cDNAs reported above were labeled and used to perform northern analysis using RNA extracted from fungal cultures grown on all of the different carbon sources used for the library construction, glucose, and incubated on no carbon source, i.e. starvation to mimic growth on a complex carbon source the fungi could not digest. Figure 4 shows these results. All of the results indicated that clones selected as negatives in the NSH and examined further by northern hybridization were indeed either not expressed or expressed only to a low extent in glucose-grown fungus, but induced to considerably higher levels in one or more of the complex carbohydrates or starved fungal cultures.

Discussion

Our goal was to isolate cDNAs induced in *A. nidulans* grown on complex carbohydrates rather than glucose and to greatly expand the number of ESTs available for *A. nidulans* without re-sequencing those generated from a glucose-grown conidiating culture (Prade et al., 2001). In the previous EST project randomly picked clones were end-sequenced and 12,320 ESTs were assembled into 4,595 contigs. Some of the clones (especially some coding for heat shock proteins) were sequenced hundreds of times reflecting their high transcript abundance. Approximately 100,000 colonies from the cDNA library made from fungus growing on complex polysaccharides were screened with probes made

from glucose-grown fungus. This allowed us to discriminate against colonies harboring cDNAs representing transcripts present in glucose-grown fungus. The screen eliminated ~92% of the colonies from further analysis. This suggests that over 90% of the messages in the complex carbohydrate-grown fungus are common to fungus grown on glucose. We expect that a large fraction of the messages are from abundant transcripts. About half of the colonies which were negative in the initial screen were not picked because of lack of separation, small size, or failure to test negative in the secondary screen. From the original 100,000 colonies plated, 3,532 were finally picked for sequencing. After processing the 3,532 resulting ESTs 2,039 unique contigs were obtained. Of these 1,772 had not been found previously within the glucose-grown conidiating library. Thus, the NSH method was very efficient in selecting for the desired cDNAs.

It had been estimated that *A. nidulans* codes for approximately 8,000 genes (Kupfer et al., 1997) although recently 9,500 ORFs have been predicted from the whole genome sequence (http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/). The combination of these two EST libraries accounts for almost 70% of the predicted transcripts.

The technique was also successful in avoiding redundancy. For example, the cDNA encoding a particular 30 kDa heat shock protein was isolated 411 times during the random screening of the cDNA library made from glucose-grown conidiating fungus whereas it was found only three times during the NSH screen. It was most likely abundant in the original cDNA library but was removed through the NSH screening procedure.

Since our library was constructed from pooled RNAs of fungal cultures grown in twelve different plant polysaccharides, the isolated cDNAs could be from one or more conditions. To test if the isolated cDNAs are expressed in the manner expected (i.e. are

induced by one or more of these polysaccharides but absent when grown on glucose) we selected unique cDNAs representing 728 contigs and hybridized them by conducting single-channel, slide-based microarray hybridization to labeled cDNAs made from fungal cultures grown on individual polysaccharides. Approximately 65% of the ESTs probed did not hybridize to labeled cDNAs from glucose-grown fungal cultures. Of these, some of the cDNAs were detected very specifically in hybridization using labeled cDNAs prepared from cultures grown on single or a few different carbon sources, but many were detected in hybridization using labeled cDNAs from a variety of cultures. This perhaps indicates a generalized shift in metabolism caused by the switch from glucose as carbon source to less readily metabolized polysaccharides with concomitant release from carbon catabolite repression which invites further study.

Although we initially designed the NSH system to find de-repressed and induced transcripts, it is clear from previous work (Nelson et al., 1999) that transcripts which are of low abundance, including those in glucose-grown fungus, could be detected as negatives in this type of screen because the corresponding labeled cDNA used to screen the library would be at too low a concentration to produce a detectable signal. Nelson et al., screened macroarrays of randomly picked colonies of a cDNA library from human prostrate tissue with labeled cDNA from the same tissue and selected the non-hybridizing colonies as rare transcripts. It is therefore likely that some of the 80 "selected negatives" which did not hybridize in our single channel microarray studies also represent rare transcripts. It could also be possible that some of the 80 non-hybridizing arrayed negatives may be transcripts specific to one of the six carbon sources used in preparing the cDNA library but not tested in the microarray analysis.

Despite our efforts to eliminate transcripts found in glucose-grown fungus, approximately 35% of the ESTs from our collection of NSH-ESTs arrayed on the glass slides hybridized to labeled cDNAs extracted from fungus grown in glucose. Of these, one-quarter hybridized strongly under all nutritional conditions whereas three-quarters hybridized more strongly in some of the complex carbon sources compared to glucose. Selection of "false-negatives" can be attributed to experimental limitations common to hybridization screening. For example, colonies may have been in a region of the membrane that was not uniformly exposed to the labeled cDNA probe or mistakes could have been made in selecting negative colonies from over-crowded plates. Repressed growth of individual colonies or incomplete transfer of colonies during membrane lifts would also result in a reduction or complete loss of hybridization signal. These types of mistakes could account for the occasional selection of cDNA clones which gave a high degree of hybridization to glucose-grown fungal cDNA in microarray experiments.

A few of the clones showing intense hybridization to glucose-grown labeled fungal cDNAs used in the microarray experiments, were picked multiple times using NSH, thus, making it unlikely that they were the result of repeated double selection during the screening. In addition, most of the false negatives were not present in the previously characterized EST collection made from a glucose-grown conidiating library. Some of the selected negative cDNA clones may have produced a weakly positive signal in northern or microarray hybridization experiments due to fairly small stretches of sequence similarity to transcripts present in glucose-grown fungus resulting in cross-hybridization. For example, in the combined EST libraries, we observed five different cDNA sequence contigs presumed to code for 30 kDa heat shock proteins, but the EST found by NSH (derived from 21 cDNA

sequences) contains about 100 nucleotide stretch with high homology to the other four heat shock proteins. We believe this would be sufficient to allow cross-hybridization with different heat shock protein transcripts from glucose-grown fungal cDNA and yield a positive signal in microarray and northern hybridizations.

The question remains as to why it behaved as a negative during the NSH experiment yet was positive in northern hybridization. The cDNA probe concentration used in the various hybridization experiments could provide another factor in the selection of false negatives in NSH. Considering that the probes were applied in the hybridization methods at the same total DNA concentration, the effective concentration of a particular cDNA probe species was much lower in the NSH hybridization as it consisted of heterogeneous mixtures containing thousands of different sequences, compared to that used in northern blots which consisted of single, homogeneous cDNA probes. Several apparent discrepancies between NSH, northern blots and microarray hybridizations may also be related to basic technical differences and limitations of these methods. Further, while NSH method worked well in this study, the potential for cross-hybridization within gene families is likely if applied to organisms with highly complex genomes.

Some of the advantages of NSH are listed: This technique should be applicable to any preferably non-normalized cDNA library as long as it contains a low percentage of empty vectors which would, of course, appear as negatives. The absence of a PCR step in the construction of the library reduces the risk of disproportionate amplification of some sequences. There are no restriction digestion steps involved during the construction of the cDNA library, which lead to small insert size. Since the library used in the NSH method is made from potentially full-length cDNAs, each negative selected has a good chance of

containing a full-length cDNA. It should be straightforward to automate most of the steps in the NSH procedure. Thus, NSH is an efficient method for isolation of cDNAs for differentially expressed and, very likely, rarely expressed transcripts.

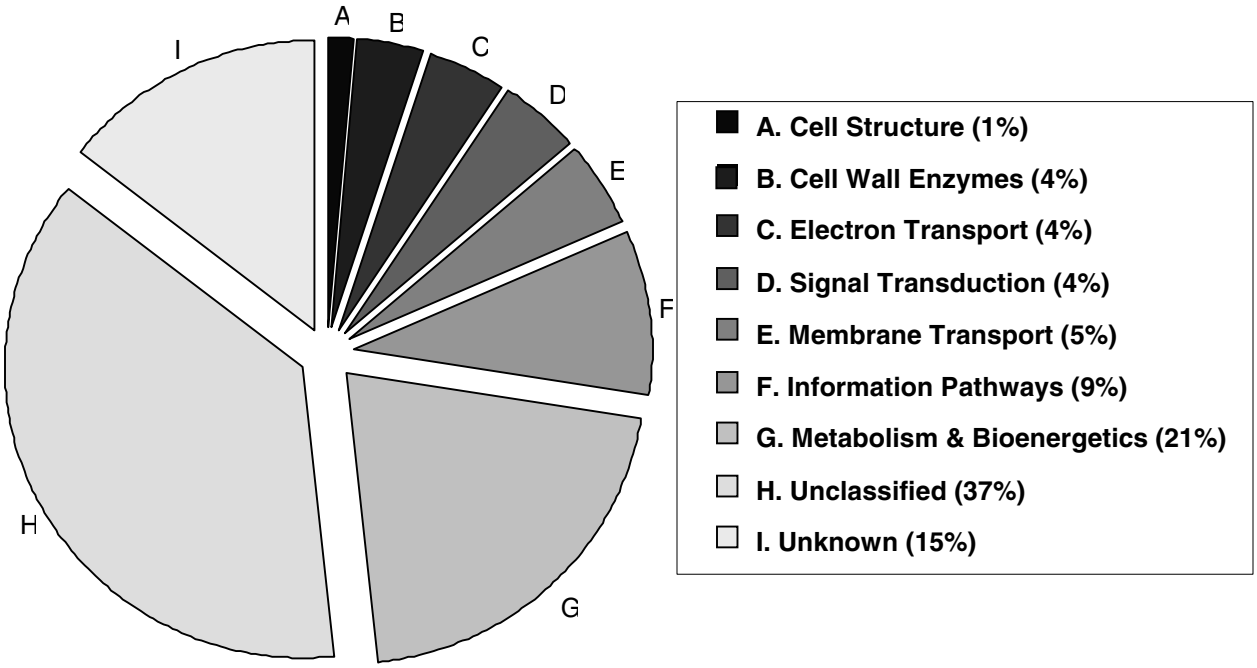

Figure 1. Functional classification of ESTs isolated by NSH

NSH-ESTs were classified into the major functional categories according to PipeOnline. The percentages indicate distribution of predicted EST functions in broad functional categories. All of the broad categories defined by PipeOnline are represented.

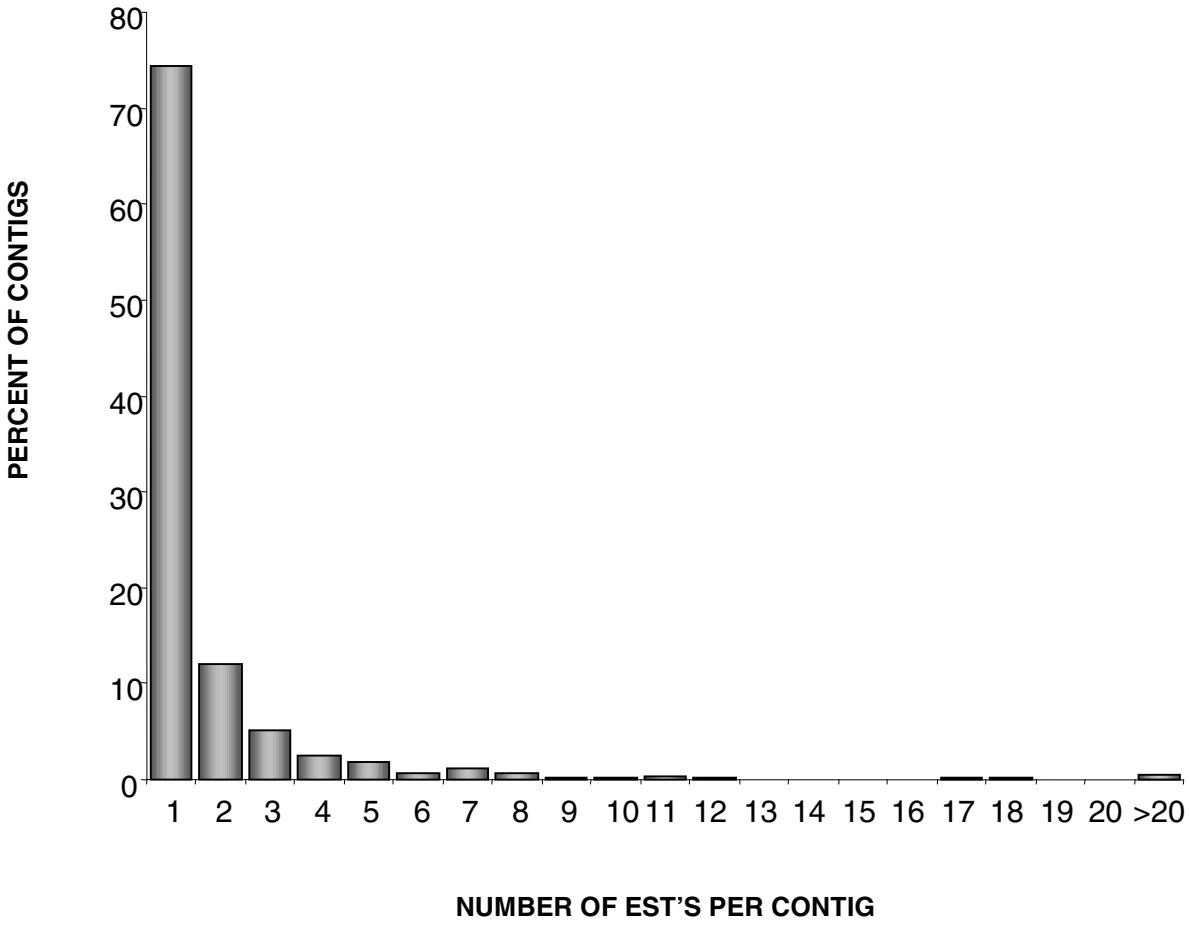

Figure 2. Bar graph showing the redundancy rates of NSH-ESTs

The NSH-ESTs were assembled into contigs and the number of ESTs per contig was determined, against which was plotted the percentage of contigs containing that number of NSH-ESTs. Seventy-five percent of the contigs are composed of one NSH-EST.

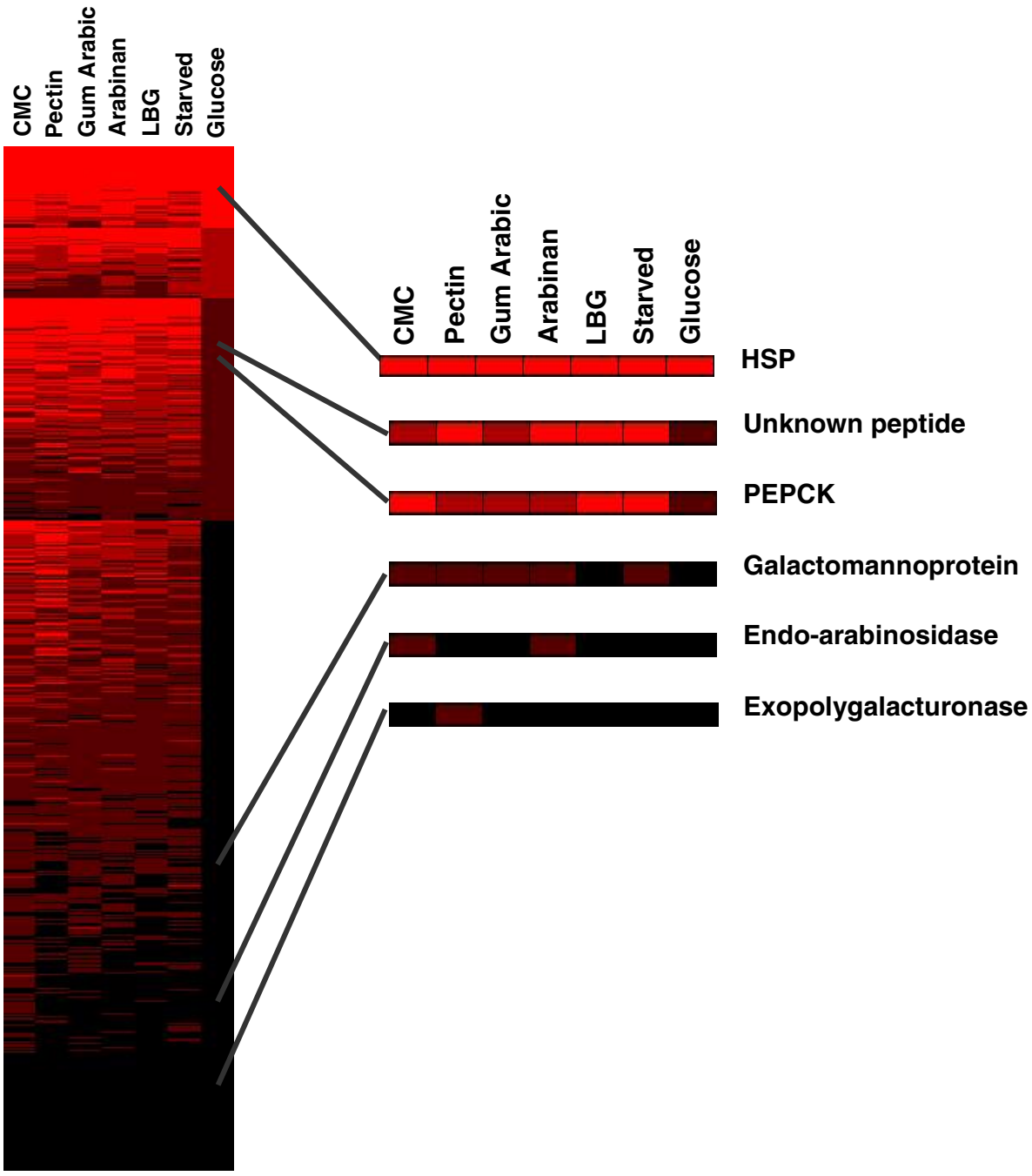

Figure 3. Heatmap representation of normalized hybridization signals

For each carbohydrate tested, background-subtracted hybridization signals were normalized between slides to the intensity of the controls and then assigned a number between 0 and 3

depending on the normalized intensity. Signal intensities of 0-4999 were assigned a number of 0 and were colored black in the heatmap; those 5000-19,999 were assigned a number of 1 and were colored light red; those 20,000-39,999 were assigned a number of 2 and were colored medium red; and any intensities above 40,000 were assigned a number of 3 and were colored bright red to assist in visualization of the hybridization signals. Signal intensities of the selected cDNAs coding for heat shock protein (HSP), unknown peptide, phosphoenolpyruvate carboxykinase (PEPCK), galactomannoprotein, endo-arabinosidase and exopolygalacturonase are shown on the right hand side of the diagram.

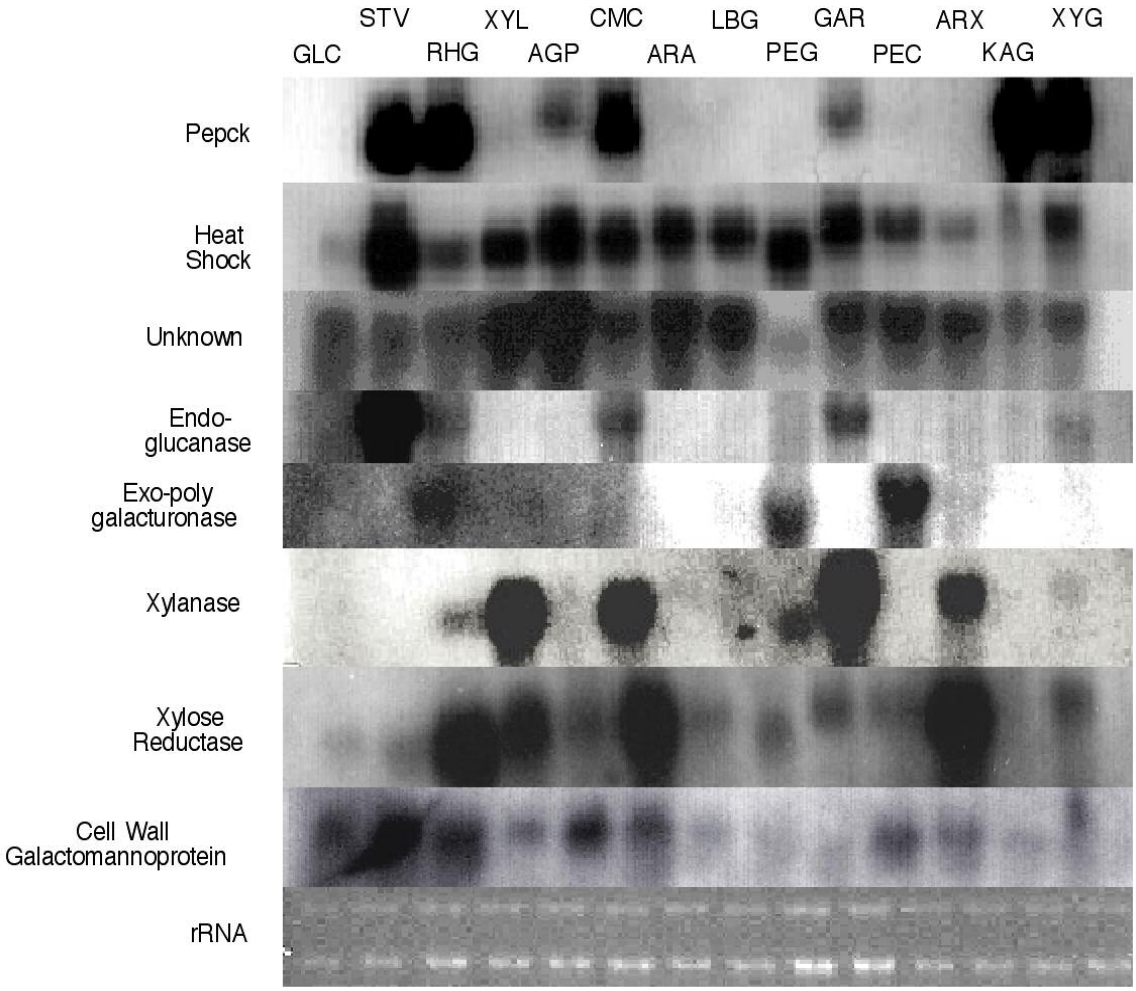

Figure 4. Northern analysis

Northern blot expression pattern of *A. nidulans* mRNAs corresponding to the cDNAs encoding (from top to bottom) phosphoenolpyruvate carboxykinase (Pepck), heat shock protein, protein with an unknown function, endoglucanase, exopolygalacturonase, xylanase, xylose reductase, and cell wall galactomannoprotein. Total RNA was isolated from fungal mycelia and ~10 µg of RNA was separated by electrophoresis on a 1% agarose/ formaldehyde gel, blotted on nylon membranes, UV cross crosslinked and hybridized to cDNA probes as indicated. The order of total RNA in the gel from left to right are: glucose plus (GLC), glucose minus or starved (STV), rhamnogalacturonan (RHG), xylan (XYL),

arabinogalactan protein (AGP), carboxy methyl cellulose (CMC), arabinan (ARA), locust bean gum (LBG), pectic galactan (PEG), gum arabic (GAR), pectin (PEC), arabinoxylan (ARX), karaya gum (KAG), xyloglucan (XYG).

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CHAPTER 3

COMPARISON OF TRANSCRIPTIONAL CHANGES IN *ASPERGILLUS NIDULANS* GROWN ON THE COMPLEX CARBOHYDRATE PECTIN OR WITHOUT A CARBON SOURCE

Introduction

When microorganisms begin a colonization cycle on plants, regardless of whether the association is pathogenic or saprophytic (i.e. biomass decays), the initial complex carbon source they come across is pectin (Prade et al., 1999). Pectins are a family of heteropolysaccharides that are comprised of predominantly 1, 4-linked α-D-galactopyranosyl uronic acid residues (Prade et al., 1999). Beyond pectin, large quantities of cellulose and hemicellulose, but little of free glucose, are available to the microorganisms while spreading through the plant tissues (Aristidou et al., 2000). Many bacteria and fungi are effective in depolymerizing and metabolizing pectin, cellulose, and hemicellulose as energy and carbon sources. Complete decomposition of the above substrates involve numerous enzymes, some of which are at least partially dependent on the action of one or more prior enzymes (Seiboth et al., 1997). The natural interactions that happen at this level are of importance to several aspects of biology and play a key role in the biotechnological utilization of biomass.

The filamentous fungus, *A. nidulans* adapts among competing microorganisms in an environment with restricted resources by adjusting to varying nutrient conditions (Flipphi et

al., 2003). The genetic components and the regulation of the genes involved in such adaptations are not very well known. Since most polysaccharide degrading fungal enzymes are present at only trace levels when only simple carbon sources (e.g. glucose) are present, little is known about the recognition mechanism that discriminates between different classes of polysaccharides.

In *A. nidulans*, enzyme specific induction and carbon catabolite repression (CCR) are two known genetic regulatory mechanisms that enable flexibility in utilizing a wide range of carbon sources. There may be common regulatory mechanisms involved in induction of enzymes for the degradation of entire classes of polymers. For example, in the presence of a metabolite of polygalacturonic acid, enzymes such as exopolygalacturonase, endopolygalacturonase, pectinmethylesterase, arabinosidase and several other enzymes could also be present. In the case of CCR, CreA, a DNA-binding transcriptional repressor becomes functional in response to repressing carbon sources. The *creA* gene from *A. nidulans* encodes a C_2H_2-zinc finger protein that binds to a 5'-SYGGRG-3' consensus sequence in the promoter region of genes subject to CCR (Strauss et al., 1999). Much is known about the target genes for *creA* binding and the involvement of *creA* in the regulation of xylan, proline, and ethanol utilization in *A. nidulans* (Orejas et al., 1999, Cubero et al., 2000, Felenbok et al., 2001) but very little is known about the processes by which CreA senses, responds, and adapts to repressing carbon sources (Flipphi et al., 2003).

The first step we took towards understanding transcriptional changes for the utilization of a complex carbon source was to isolate a nearly complete set of genes potentially involved in the process. We devised a Negative Subtraction Hybridization (NSH) procedure (Ray et al., 2004) to isolate cDNAs for a large group of genes that are induced by

switching glucose-grown fungal mycelia to one of twelve different complex polysaccharides. The twelve polysaccharides represent almost all structural polysaccharide structures present in plant tissues. Analysis of the isolated cDNAs by sequence homology indicated a generalized shift in metabolism caused by the switch from glucose as carbon source to less readily metabolized polysaccharides.

Here we describe the change in expression profiles of mRNAs when the fungus is withdrawn from glucose and instead provided an alternative complex substrate, pectin, which we expected would provide release from carbon catabolite repression and/or induction. We also describe the effect of glucose starvation (no carbon source), which we expected would result in release from carbon catabolite repression in the initial stages of incubation on minimal medium devoid of any carbon source followed by upregulation of genes involved in survival. Our specific objectives were: 1) to identify transcripts that are upregulated or derepressed in pectin only but not in glucose starvation, 2) to identify transcripts that are upregulated or derepressed in glucose starvation only but not in pectin, and 3) to identify transcripts that show a "shared response" i.e. upregulated or derepressed by pectin and glucose starvation.

Xie et al., in previous studies with *Neurospora crassa* (*N. crassa*), reported transcriptional profiling of glucose-grown cultures compared to glucose-starved cultures (Xie et al., 2004). *N. crassa* is known to ferment glucose to ethanol in aerobic culture (Colvin et al., 1973) whereas *A. nidulans* is known to utilize glucose primarily through respiration with low levels of fermentation (Kelly et al., 1990, Lockington et al., 1997).

This is the first study in *A. nidulans* designed to monitor the transcriptional response to glucose starvation and pectin using microarray analysis. Our results revealed changes in

transcript levels for about 32% of the cDNAs printed on our arrays after a switch in carbon source from glucose to glucose starvation and /or pectin.

Materials and Methods

Strain, media and culture conditions

A. nidulans FGSC C26 strain was used in all experiments. Minimal and complete media with appropriate supplements were prepared as suggested by Kafer (Kafer E 1977) and manipulated as described by Pontecorvo and co-workers (Pontecorvo et al., 1953). A two stage vegetative growth and forced carbon shift induction system was used where vegetative mycelia were initially produced by inoculating 1×10^8 conidia per ml in minimal medium supplemented with 1% glucose and incubated at 37°C, pH 6.5 with continuous shaking at 200 rpm for 18 h. Mycelia were recovered by filtration, washed with sterile water, and transferred to fresh minimal medium containing no glucose, 1% glucose or 1% pectin (Sigma Chemical Co., St. Louis, Mo.), and grown for 3 h, 6 h, 9 h, and 12 h at 37°C, pH 6.5 with continuous shaking at 200 rpm. For examination of the fungal growth in glucose, pectin, and no carbon source (i.e. glucose starvation), 20 ml of the samples were withdrawn using sterile pipette tips at 2 h intervals over a period of 12 h and filtered through pre-weighed filter papers. The filter papers were dried in a freeze drier for 16 h and the dry weight of the mycelia was determined. The culture filtrates were collected and kept frozen at -20°C in sterile 50 ml corning tubes for further analysis.

HPLC Gel filtration chromatography

For examination of the degradation of pectin by the fungus, 20 ml of the culture filtrates were withdrawn at 2 h intervals over a period of 12 h. Aliquots of 1 ml of the culture medium were fractionated by gel filtration on a column of Toyo pearl HW 40(S) from Supelco Inc (fractionation range for carbohydrates was from 100-7,000 molecular weight). Beads were packed in a steel column (10mm×500mm) and the column was equilibrated with 50mM ammonium acetate, pH 5.2, with a flow rate of 1.0 ml/min. The sugars were detected by refractive index (SHODEX R1-71).

Capillary Zone Electrophoresis (CZE)

Glucose levels remaining in the culture filtrates were determined by CZE. An aliquot of the medium (10 µl) was mixed with 10 µl of 20 mg/ml cellobiose as an internal standard and diluted 100 fold. A 2 µl aliquot of this was derivatized with aminonaphthalene trisulfonic acid (ANTS) by heating it at 80^{o}C for 1 h with 10 µl of 30 mM ANTS in 3% acetic acid and 1 µl of 1M $NaCNBH_3$ in dimethylsulfoxide (Zhang et al., 1996). The resulting solution was analyzed by CZE using a custom built instrument, and the electrophoresis conditions reported previously (Merz et al., 1998). The ratios of peak areas for the glucose to cellobiose were compared to that obtained from a standard mixture of a 10 mg/ml glucose solution with cellobiose. Galacturonic acid levels in the medium of pectin grown fungus were determined the same way, except that 2 µl of the undiluted medium and 2 µl of 100 fold diluted cellobiose solution were directly derivatized. Oligosaccharides could also be observed (Mort et al., 1996), but not quantitated because of lack of separation and necessary standards.

Microarray analysis

For expression profiling using microarray analysis, *A. nidulans* was grown as mentioned above. Cultures for each time point and condition tested were grown in quadruplicate and the tissues were harvested, blotted dry, frozen and lyophilized prior to RNA isolation. The procedure for printing of the slides, the probes, and the treatment of the slides were exactly as previously reported (Ray et al., 2004).

Preparation of labeled cDNAs and the hybridization procedure

Total RNA was isolated from *A. nidulans* grown in pectin or glucose-starved condition using TRIZOL reagent (Sigma Chemical Co., St. Louis, Mo.). The quality of RNA samples was assessed by electrophoresis using a denaturing formaldehyde/agarose gel. RNA samples with an A_{260}/A_{280} ratio between 1.8 and 2.0 having intact ribosomal RNA bands were selected for use in preparing labeled cDNAs for hybridization. Twenty-five micrograms of total RNA was used for labeling using Genisphere's Array 350 hybridization kit according to the manufacturer's instructions. Total RNA samples from each time point from glucose-grown fungus were labeled with Alexa 546 and used as reference and total RNA samples from pectin-grown fungus at each corresponding time point were labeled with Alexa 647 and used as experimental RNA. Similarly, for the glucose-starved experiments, the reference was the Alexa 546 labeled RNA from glucose-grown fungus, and the experimental sample was Alexa 647 labeled RNA isolated from glucose-starved fungus at each time point. Replicated RNA samples from each time point were pooled before labeling and hybridization. Each hybridization for a particular condition tested per time point was repeated two times with pooled total RNA samples from four replicates of each time point. There were four spots per cDNA on each slide thereby yielding eight data points per cDNA

for each condition and time tested. The hybridization and the wash procedure were according to the manufacturer's instructions. The arrays were scanned using a Scan Array Express from Perkin-Elmer.

Data analysis from the scanned slides

The images were processed using GenePix Pro 4.0 (Axon Inc.). Pre-processing of data was accomplished using GenePix Autoprocessor (GPAP) (http://darwin.biochem.okstate.edu/gpap/) (Ayoubi et al., unpublished results). This analysis included: 1) exclusion of data where the fluorescence signal intensity in both channels was less than the background plus two standard deviations; 2) exclusion of data where the signal in both the channels was less than 200 Relative Fluorescence Units; 3) exclusion of spots which failed quality control and were flagged during processing of the images using GenePix Pro; 4) $\log_2$ transformation of the background subtracted Alexa 647/Alexa 546 median ratios. After pre-processing, the expression results were normalized using print tip LOWESS normalization (Yang et al., 2002) and a statistic was calculated for each gene based on the Empirical Bayes approach using the R-project statistical environment (http://www.r-project.org) with the Bioconductor (http://www.bioconductor.org) and Limma (Smyth et al., 2004) packages through the GPAP web site (http://darwin.biochem.okstate.edu/gpap/) (Ayoubi et al., unpublished results).

The EST sequences of all the probes used for these microarray studies have been submitted to NCBI dbEST database with accession numbers ranging from CK445320-CK449149 and CK468506-CK468532. All the microarray data are deposited at the NCBI

Gene Expression Omnibus (GEO) under the platform accession number GPL 566 and the series accession number GSE 2417.

Detection of exopolygalacturonase activity

The filtrates from the fungal cultures grown for 3 h, 6 h, 9 h, and 12 h were used to assess exopolygalacturonase activity using CZE (Zhang et al., 1996). Oligosaccharides of ten α 1-4 linked galacturonic acid residues were purified (Nothnagel et al., 1983), labeled with aminopyrene trisulphonic acid (APTS) (Evangelista et al., 1995), desalted by gel filtration chromatography, and used as a substrate for detection of oligogalacturonan degrading activities. The labeled substrate was diluted to about 100 ng/µl with water and 1 µl of it was mixed with 1 µl of the culture filtrate and buffered with 18 µl of sodium acetate buffer pH 4.0. After incubation for 5 min, 15 min, or 1 h at 37^{o}C the enzyme was heat killed at 90^{o}C for 10 min. The products were subjected to CZE on a BioRad Biofocus 2000 instrument with excitation by a 488 nm argon-ion laser with fluorescence collected through a 520 nm bandpass filter. Electrophoresis was conducted at 15 KV in 0.1M phosphate buffer, pH 2.5 on a 31 cm 50 µ ID capillary.

Results

Physiological response of the fungus to changes in carbon source

The effect of a switch in carbon source on fungal growth was investigated by following the change in dry weight of the mycelia through a 12 h growth period. In parallel, we followed the disappearance of glucose and pectin from the culture filtrates. The dry weight of fungal mycelia per 20 ml of culture was determined at 2 h intervals after the

transfer to the test medium. Figure 5 shows that, as expected, the fungal dry weight gradually declined in glucose-starved condition. In pectin, the fungal dry weight was maintained in the initial time points then showed an increase after 8 h while in glucose, the fungal dry weight increased slowly.

The decline in the amount of pectin in the medium of pectin-grown fungus was estimated by passing an aliquot of the medium through a gel permeation column and integrating the area of the peak eluting in the void volume. Fragments of pectin fewer than about 5 sugar residues would elute later than the polymer peak. Chromatography of a corresponding aliquot of medium from glucose-grown fungus gave only a very small peak in the void volume, suggesting that the area of the void volume peak from the pectin-grown fungus truly reflects the amount of pectin polymer. After about a 3 h lag, the concentration of pectin declined rapidly (Figure 5 inset). We could estimate the concentration of free galacturonic acid and oligomers of it by labeling an aliquot of the medium with aminonaphthalene trisulfonic acid (ANTS) and then analyzing the products with capillary zone electrophoresis (data not shown). For the first 6 h free galacturonic acid could not be distinguished among the background of other minor components in the medium. However, at 6 h the level of free galacturonic acid rose to about 1 mg/ml and remained at that level for the remainder of the experiment (data not shown). There was no apparent accumulation of the short GalA oligomers that are the end products of digestion of homogalacturonan by endopolygalacturonases. However, we could see an accumulation of longer more complex oligomers that appeared to become shorter as time progressed. The fungus did not increase in dry weight until 8 h indicating that it took several hours to switch its metabolism to use galacturonic acid presumably as a source for making glucose required for its growth.

The concentration of glucose in the medium of the glucose-grown fungus was determined by labeling an aliquot of the medium with ANTS and subjecting it to CZE. After the transfer to growth on glucose the fungal dry weight slowly increased concomitant with a steady uptake of glucose from the medium (Figure 5 inset).

Gene expression analysis

We chose transcriptional profiling using microarrays to observe the effect of derepression and/or induction and to monitor those genes which play a key role during the growth of *A. nidulans* when shifted from growth on glucose to growth on pectin or glucose-starved conditions. We collected mycelium at various time points of incubation of *A. nidulans* following the medium shift as outlined in materials and methods. Based on the observed loss of pectin from the cultures combined with the increase in dry weight, we chose four different time points of growth i.e. 3 h, 6 h, 9 h, and 12 h after shifting the carbon source to pectin and glucose-starved conditions (arrows in Figure 5). Total RNA was extracted from the pooled tissue at each time point as mentioned above, labeled and hybridized to the microarrays. The raw data were processed using the GPAP program (http://darwin.biochem.okstate.edu/gpap/) and the output files were used for further analysis. We used a stringent method of B-statistics based on the Empirical Bayes approach to generate a list of 312 genes which had at least a two-fold change in expression ($\log_2$ ratio of 1) and a B-statistic value of 1 or greater at one or more of the time points under the conditions tested.

These 312 genes were placed into three different categories. 1) Those with a glucose starvation specific response were significantly up or downregulated in at least one of the

times tested under the glucose-starved condition but not in pectin-grown condition, 2) Those with a pectin-specific response were significantly up or downregulated in at least one of the times tested in pectin-grown condition but not in glucose-starved condition, and 3) Those with a shared response were significantly up or downregulated in both conditions, in at least one of the times tested, but not necessarily at the same time.

Figure 6 shows a bar graph depicting the distribution of pectin-regulated or glucose starvation-regulated genes. The gray segments reflect the number of genes that were upregulated or downregulated at each time point in either glucose-starved condition (panel A) or pectin-grown condition (panel B) at 3 h, 6 h, 9 h, and 12 h but not under both conditions. Hence, these genes are examples of condition specific regulation. The white segments reflect the numbers of genes showing a shared response that were upregulated or downregulated at each time point under both glucose-starved condition and pectin-grown condition (i.e. shared response).

Hierarchical cluster diagrams of the three different categories of differentially expressed genes are shown (Figure 7). They are divided into three panels A, B, and C depending on whether they depict genes which respond to only glucose starvation, pectin or both glucose starvation and pectin. Panel A depicts the hierarchical cluster diagram of the 109 genes which show a glucose starvation specific response at any one of the time points. Of these, 62 genes have no known function (BLASTX search, E-value $\leq$ 1e-20). These genes are probably examples of derepression by release from carbon catabolite repression. We observed 89 genes with a pectin-specific response (panel B) out of which 38 genes have no known function (BLASTX search, E-value $\leq$ 1e-20). Finally, there are a total of 114 genes with a shared response (panel C) out of which 56 genes have no known function

(BLASTX search, E-value ≤ 1e-20). Table I lists the genes grouped into different metabolic pathways and other functional categories.

Polymer degradation and sugar transport

Presumably the complex polysaccharide pectin is degraded and utilized by *A. nidulans* using several different enzymes and pathways. We expected some enzymes will be expressed as a result of derepression by release from carbon catabolite repression while others will be specifically induced in the presence of a complex carbon source like pectin. The backbone of the pectin chain, predominantly α (1-4) galacturonic acid (GalA), is degraded by polygalacturonases and lyases into oligomers and then degraded to monomers for uptake by the fungus. Exopolygalacturonases cleave one residue at a time from the non-reducing end of polymers and oligomers. High amounts of exopolygalacturonase activity were detected by capillary electrophoresis of the culture filtrate from fungus growing on pectin for 3, 6, 9, and 12 hours (data not shown). Correspondingly, a transcript encoding an exopolygalacturonase was significantly upregulated in pectin-grown cultures at all the time points (10.7-fold, 3.0-fold, 4.3-fold, and 3.9-fold) but not under glucose-starved condition. In contrast, transcripts encoding arabinosidase (an enzyme involved in the degradation of the side chains of pectin), xylosidase and alpha-mannosidase did not show any significant change in expression in pectin-grown cultures. This suggests the need for both induction and derepression.

Out of the seven predicted sugar transporters (AN6804.2, AN6669.2, AN2466.2, AN2794.2, AN4148.2, AN8400.2, and AN2585.2) included on the microarray, transcript levels for four of these revealed a shared response with elevation in glucose-starved

condition at least initially and in pectin-grown condition towards the later times. The expression level of AN6669.2 (clone Set1P5D6), which encodes a high-affinity sugar transporter subject to carbon catabolite repression (vanKuyk et al., 2004), was upregulated at 3 h (3.6-fold) in glucose-starved condition and at 9 h (4.0-fold) and 12 h (4.8-fold) in pectin-grown condition.

The expression level of AN6035.2 (clone Set1P3F8) was upregulated in pectin-grown cultures at 9 h (3.8-fold) and at 12 h (4.2-fold) but was not significantly expressed in the glucose-starved cultures. A domain located within the predicted peptide sequence (http://www.broad.mit.edu/annotation/fungi/aspergillus/) suggests this protein belongs to a family of muconate lactonizing enzymes which function in the beta-ketoadipate pathway used in the catabolism of aromatic compounds in fungi and yeasts (Mazur et al., 1994).

Glycolysis

Seven transcripts encoding glycolytic enzymes were on our arrays. The transcripts encoding hexokinase, phosphofructokinase, and alcohol dehydrogenase III did not show any significant change in expression at any of the times tested under the glucose-starved condition or the pectin-grown condition. Transcription of the gene encoding phosphoglycerate kinase was downregulated in glucose-starved condition while the transcription of fructose bisphosphate aldolase was upregulated (2.7-fold) only at 12 h in the glucose-starved condition and did not show any change in expression in the pectin-grown cultures. The transcripts from the glycolytic pathway that were significantly upregulated at 9 h and 12 h in glucose starvation and in pectin-grown condition were glyceraldehyde-3-phosphate dehydrogenase (AN8041.2) and enolase (AN5746.2). The expression of

glyceraldehyde-3-phosphate dehydrogenase was upregulated in glucose-starved cultures at 9 and 12 hours (3.1-fold and 2.3-fold, respectively) and in pectin-grown cultures at 9 and 12 hours (2.4-fold and 2.1-fold, respectively). The expression of enolase was upregulated 3.1-fold and 2.2-fold in glucose-starved cultures at 9 and 12 hours, respectively, and 2.2-fold and 3.8-fold in pectin-grown cultures at 9 and 12 hours, respectively.

Gluconeogenesis/Pyruvate branchpoint

Previous work in yeast has shown that the degradation of mRNA for phosphoenol pyruvate carboxykinase (PEPCK) involved in gluconeogenesis can be triggered by < 0.02% glucose concentration in its medium (Yin et al., 2000). PEPCK (AN1918.2) converts oxaloacetate to phosphoenolpyruvate and plays a key role in gluconeogenesis. In our study, transcription of PEPCK was significantly upregulated (4.7-fold, 4.9-fold, 5.5-fold, and 3.6-fold) at all of the times during growth under glucose-starved condition and at 3 h (2.2-fold) in pectin-grown condition. The transcription of the gene encoding pyruvate dikinase (AN5843.2) was significantly upregulated in glucose-starved condition at 3 h (2.1-fold), 6 h (4.8-fold), 9 h (3.3-fold), and 12 h (3.4-fold) and initially in pectin-grown cultures (2.3-fold). Pyruvate dikinase catalyzes the reversible conversion of pyruvate to phosphoenolpyruvate. Interestingly, the expression pattern of pyruvate carboxylase was not significantly changed under either glucose-starved or pectin-grown condition. The gene expression pattern of acetyl-coenzyme A synthetase which activates acetate to acetyl-CoA showed upregulation on pectin-grown cultures towards the later times but did not show any change in its expression under glucose-starved condition. Acetyl-CoA is effectively carried into the mitochondria by a shuttle mechanism in which carnitine acetyltransferase

(AN6279.2) plays a key role (Stemple et al., 1998), and the expression of this gene increased 3.4-fold in glucose-starved condition and 2.6-fold in pectin-grown condition.

TCA cycle

Several transcripts for TCA cycle enzymes on our arrays showed interesting expression patterns. The transcript encoding ATP-dependent citrate synthase (AN2436.2) was downregulated under both the glucose-starved and pectin-grown conditions. The expression level of succinate dehydrogenase did not show any significant differential expression in glucose-starved and pectin-grown fungal cultures. However, transcripts including isocitrate dehydrogenase (AN2999.2), oxoglutarate dehydrogenase (AN5571.2), and succinyl-CoA synthetase (AN2295.2) were upregulated at one or more times in either the pectin-grown or the glucose-starved condition or in both the conditions. The expression of AN2999.2 was upregulated during 6 h (2.0-fold), 9 h (3.8-fold), and 12 h (4.0-fold) of growth under glucose-starved condition and during 3 h (7.8-fold), 9 h (2.1-fold), and 12 h (2.9-fold) of fungal growth in pectin. Isocitrate dehydrogenase catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate, which can lead to the synthesis of glutamate. This reaction is the rate-limiting step of the TCA cycle and is an important branchpoint between catabolic and anabolic processes (Szewczyk et al., 2001). Transcripts for glutamate synthase and glutamine synthase were upregulated in the pectin-grown cultures but did not show any significant change in expression in the glucose-starved cultures. Transcription of the gene encoding glutamate dehydrogenase did not show any significant change in expression under glucose-starved condition but was downregulated in pectin-grown condition. The expression level of AN4869.2, which encodes a proteasome subunit involved

in protein catabolism, was upregulated in glucose-starved condition but repressed in pectin-grown condition. Expression of genes (AN0913.2, AN6731.2, AN4135.2, AN1176.2) (Table 1) involved in lipid metabolism were downregulated in glucose starvation as compared to glucose-grown cultures.

Electron transport chain and oxidative phosphorylation

Alternative oxidase (AN2099.2) showed an interesting expression pattern in glucose starvation and in pectin grown condition. In our studies, the transcription of alternative oxidase was upregulated in the initial stages of the fungal growth under glucose-starved condition but did not show any significant difference in expression in the later stages. In the case of fungal growth on pectin, the alternative oxidase cDNA was upregulated at 3 h (3.3-fold) but then downregulated at 9 h and 12 h (-3.8-fold and -5.5-fold, respectively) as compared to the glucose-grown condition. Alternative oxidase is a second oxidase (in addition to cytochrome c oxidase) present in mitochondria of plants, fungi, protists, and yeasts (Lorin et al., 2001). Alternative oxidase receives electrons from ubiquinone and catalyzes the direct reduction of oxygen to water, thereby circumventing Complex III and Complex IV and hence, two of the proton pumping steps. In contrast to alternative oxidase, the transcript for cytochrome c reductase (Complex III) was upregulated at 9 h (2.1-fold) and 12 h (3.0-fold) in pectin-grown cultures. Also, the gene encoding a subunit of ATP synthase was upregulated at 9 h (2.0-fold) and 12 h (2.6-fold) in pectin-grown fungal cells. ATP synthase utilizes ADP and Pi in the endergonic synthesis of ATP which is needed for cellular growth. Additionally, a gene encoding ADP/ATP translocase was upregulated at 9 h (2.0-fold) in pectin-grown fungal cells but downregulated at 3 h in glucose-starved fungal cells.

ADP/ATP translocase catalyzes the ADP/ATP exchange across the mitochondrial membrane. This is the terminal step of mitochondrial oxidative phosphorylation (Nelson et al., 1998).

Cell wall biogenesis

A gene (AN7657.2) encoding a β (1-3) glucanosyltransferase was found to be upregulated at 9 h and 12 h in pectin-grown fungus (2.3-fold and 1.9-fold, respectively). In previous studies on a homologous glucanosyltransferase from the opportunistic fungus *A. fumigatus*, the enzyme was shown to play an active role in the biosynthesis of the fungal cell wall during growth (Mouyna et al., 2000). It first cleaves a β (1-3) glucan molecule present in the cell wall and then transfers the newly generated reducing end to the non-reducing end of another molecule of β (1-3) glucan, thereby forming a new linkage and resulting in elongation of the β (1-3) glucan chain. Also, the transcript (AN8484.2) for a cell wall galactomannoprotein was upregulated at 9 h and 12 h in pectin-grown cultures. There have been reports regarding fungal cell wall structure indicating that mannoproteins are important components of the cell wall and galactomannoproteins have been isolated from the cell wall of *A. fumigatus* (Woo et al., 2002).

Another gene (AN8244.2) encoding a spindle pole body-associated protein (SNAD) was upregulated (2.0-fold and 2.1-fold) in pectin-grown cultures towards the later times (9 h and 12 h, respectively). SNAD is likely to be involved in temporal regulation of septation in *A. nidulans* (Liu et al., 2000).

Transcription factors

Some of the transcription factors showed interesting expression patterns in pectin-grown as well as glucose-starved fungal cells. *xlnR* (AN0388.2), encoding a transcriptional activator coordinating xylanolytic enzyme expression, was found to be upregulated at 9 h (2.0-fold) and 12 h (1.9-fold) in pectin but did not show any significant difference in expression at the early hours of the pectin-grown cultures or at any stage of growth under glucose-starved condition. The observed expression pattern of *xlnR* could be due to trace amounts of D-xylose in the commercial citrus pectin used in our experiments (Zhan et al., 1998). There have been reports indicating that only trace amounts of xylose are sufficient to induce *xlnR* in *A. niger* (van Peij et al., 1998). Another transcriptional activator, *cpcA* (AN3675.2), belonging to the family of c-Jun-like transcriptional activators, was upregulated in glucose starvation but did not significantly change its expression when the fungus was grown in pectin. In *A. nidulans* it has been shown that CpcA acts as a central transcription factor in the "cross pathway control" network that regulates the synthesis of amino acids (Hoffman et al., 2001). Such networks become activated under amino acid starvation. *creA* (AN3179.2), encoding a DNA-binding regulatory protein, which plays a key role in carbon catabolite repression, was upregulated during growth of the fungus on pectin as well as under glucose-starved condition.

In silico promoter analysis

The region 1 kb upstream of the predicted translational start site of the genes that were upregulated in pectin only and upregulated in glucose starvation only were analyzed for the occurence of the putative CreA binding site of a pair of [5'-(G/C) (C/T) GG (G/C) G-3']

sequences, where the pairs are separated by a spacer nucleotide sequence (3). The analysis was accomplished using the Motif-finder-auto program of the cDNA microarray and promoter analysis tool-kit (Shah et al., 2003). A *p*-value was calculated by the Motif-finder-auto program to obtain the statistical significance of the frequency of occurrence of the particular motif in the upregulated groups of genes when compared to the entire genome. The CreA binding motif was found in 16 genes out of 62 upregulated in pectin only at any time point but not in glucose starvation and had a *p*-value of 0.000978. The CreA binding motif was found in the promoter region of 21 genes out of 82 upregulated in glucose starvation only with a *p*-value of 0.00702. Considering that one-quarter of the genes in the above groups were likely influenced by CreA, it appears likely that some other regulatory mechanisms exist, apart from carbon catabolite repression, that play a regulatory role in the induction of genes under low glucose conditions.

Discussion

Our aim was to describe the transcriptional differences between fungal cultures grown on glucose and cultures grown on pectin by following the progression of changes occurring after shifting cultures from growth on glucose to growth on pectin compared to shifting from growth on glucose to growth on fresh glucose-containing medium. Since it is known that carbon catabolite repression is relieved under low glucose conditions, and it was likely that the fungus would be effectively carbon starved after the transfer to pectin until pectin degrading enzymes and enzymes for metabolism of the resulting sugar units had been produced, we also compared the transcription of the fungus switched to a medium lacking a carbon source (i.e. glucose starvation) to that of fungus grown on glucose. The physiological

experiments indicate that there is about a 6 h lag before monomeric galacturonic acid accumulates in the medium of the pectin-grown fungus, so the fungus growing on pectin is essentially as carbon starved as the fungus with no carbon source (glucose starvation) to that point.

The initial response (3 h) of the fungus to glucose starvation was an increase in transcription of a little over 60 genes, represented on our arrays of which at least 50% are upregulated during glucose starvation but not during growth in pectin. Some of the identifiable glucose starvation-specific genes encode transcription factors, or proteins which interact with transcriptions factors, and probably initiate dramatic changes in the fungus required for its survival under glucose-starved condition. The upregulated genes that are common to both glucose-starved and pectin-grown conditions are mostly associated with sugar uptake and carbohydrate metabolism. Genes required for production of enzymes involved in gluconeogenesis along with an alternative oxidase are also upregulated.

In the case of switching to growth on pectin, only 20 genes present on our arrays were upregulated after the initial 3 h, with only 6 being specific to growth on pectin. Not surprisingly, the most highly upregulated gene coded for a pectinase. Only a small proportion of genes upregulated rapidly in glucose starvation were also upregulated in pectin-grown fungus, suggesting that the fungus does indeed recognize the pectin and responds by producing pectin-degrading enzymes rather than making dramatic changes needed to survive prolonged carbon deprivation.

At 6 h the glucose-starved cultures continued to show enhanced expression of various transcription factors and enzymes for gluconeogenesis, but over half of the genes expressed at elevated levels at 3 h had returned to expression levels found in glucose-grown fungus at 6

h. The same situation held for pectin-grown fungus. Unfortunately most of these genes have not yet been ascribed a function.

After 9 h galacturonic acid levels had risen to ~1 mg/ml in the culture medium and presumably all of the enzymes needed for utilization of galacturonic acid as a carbon source had been produced. Over 20 genes specific to growth on pectin were upregulated along with another 20 upregulated by both pectin and glucose-starved conditions. Almost an equal number of genes were expressed at significantly lower levels than found in glucose-grown fungus and most of these have not yet been ascribed a function. Quite a few of the genes regulated similarly in glucose starvation and pectin-grown condition were strongly upregulated at 3 h and then downregulated at 9 h and 12 h.

At 12 h the gene expression levels in pectin-grown fungal cultures were about the same as at 9 h, indicating that the fungus had adapted satisfactorily to growth on pectin, whereas the starving cultures had increased the numbers of both upregulated and downregulated genes.

Previous studies have shown that unlike *S. cerevisae* (Wiame et al., 1985), *A. nidulans* can use amino acids as its sole carbon source (Hynes et al., 2002). The significant upregulation of the gene encoding PEPCK under glucose-starved condition is consistent with that view since PEPCK plays a key role in gluconeogenesis using non-carbohydrate precursors (amino acids) for synthesis of glucose. It would be futile from an energy point of view for any organism to carry out glycolysis and gluconeogenesis at the same time; hence, from our results, we infer that the fungus carries out gluconeogenesis to survive in the absence of glucose and in the initial stages of growth on pectin, which mimics the glucose-starved condition. Most of the transcripts encoding enzymes of the glycolytic pathway that

were on the arrays were not altered in expression in pectin-grown or glucose-starved condition except for transcript levels of glyceraldehyde-3-phosphate dehydrogenase and enolase, which were significantly upregulated in both the above conditions. These two enzymes are shared between glycolysis and gluconeogenesis, and based on our observation, we infer that these two enzymes play an active role in gluconeogenesis under the conditions tested. Another transcript (AN5843.2) upregulated in glucose starvation which is likely to play an active role in gluconeogenesis is pyruvate water dikinase. The expression level of AN5843.2 (clone N11GD2) was significantly upregulated at all times tested in glucose-starved condition and initially in pectin-grown cultures. The predicted peptide sequence of AN5843.2 has putative domains for pyruvate binding, phosphoryl group transfer and phosphoenolpyruvate binding (http://www.broad.mit.edu/annotation/fungi/aspergillus/) often found associated with pyruvate dikinase activity (Pfam). Pyruvate dikinases are involved in pyruvate metabolism and carbon fixation catalyzing the conversion of pyruvate to phosphoenolpyruvate. Interestingly, homology searches of available fungal protein sequences (BLASTP of the NBCI nr database) and public fungal EST databases (TBLASTX of NCBI dbEST) revealed only one predicted homologous protein in the *Gibberella zaea* genome and no homologous ESTs in *G. zaea* or in any fungal species other than *A. nidulans*. Homology searches of peptide sequence from the NCBI nr database (BLASTP, E-value $\leq$ 1e-20) suggest this protein is more similar to the bacterial pyruvate water dikinases (phosphoenolpyruvate synthases) as opposed to the pyruvate phosphate dikinases of plants. The expression of this gene under the conditions of glucose starvation and in the early hours of growth on pectin as a carbon source suggests a role for it in gluconeogenesis. In silico analysis of the promoter region beginning at 1 kb upstream of the genes encoding pyruvate

water dikinase and phosphoenolpyruvate carboxykinase revealed both *creA* and *cpcA* binding sites involved in carbon catabolite repression and regulation of amino acid synthesis respectively.

creA in the presence of glucose is also known to repress the *alcA* gene encoding alcohol dehydrogenase I in *A. nidulans* (Cubero et al., 1994, Flipphi et al., 2001). There are three alcohol dehydrogenase genes in *A. nidulans* capable of utilizing ethanol as a substrate (Jones et al., 2001), of which only alcohol dehydrogenase III (*alcC*) gene was present on our array and this gene did not show any significant change in expression. This result would be expected since *alcC* is preferentially transcribed under anaerobic conditions (Kelly et al., 1990). Microarray studies conducted in another filamentous fungus, *A. oryzae* showed that the alcohol dehydrogenase I (*adhA*) gene was upregulated in glucose-abundant condition and downregulated during glucose-starved condition and *adhA* from *A. oryzae* shows high similarity to both *alcC* (E-value 10^{-151}) and *alcA* (E value 10^{-150}) of *A. nidulans* (Maeda et al., 2004).

In the absence of glucose (glucose-starved or pectin-grown condition), most of the transcripts of the TCA cycle tested were upregulated relative to the glucose-grown condition, indicating TCA cycle genes are possibly derepressed to provide carbon intermediates for gluconeogenesis to generate glucose. The transcript levels of genes involved in protein catabolism under glucose-starved condition and initially in pectin were significantly upregulated as compared to glucose-grown cultures. Amino acids can enter into the TCA cycle at several branchpoints, ultimately providing intermediates for gluconeogenesis. The gene encoding the transcriptional activator *cpcA*, was upregulated under glucose-starved condition and its expression was most likely upregulated due to amino acid starvation. At the

later times transcript levels of glutamate synthase and glutamine synthase increased in pectin-grown cultures but not in glucose-starved cultures. This indicates a shift towards synthesis of amino acids in pectin-grown cultures, which correlates with a decrease in transcription of gluconeogenic genes.

A transcript for alternative oxidase was upregulated initially in glucose-starvation and pectin-grown conditions but became downregulated towards the later times in pectin-grown cultures. It has been hypothesized that the alternative oxidase prevents a buildup of high levels of reductant that would otherwise lead to the production of damaging amounts of reactive oxygen species. Alternative oxidases are believed to act as alternative terminal oxidases bypassing two proton-translocating sites associated with ATP production thus allowing turnover of the citric acid cycle intermediates (Vanlerberghe et al., 1997). In later stages of *A. nidulans* growth in pectin this alternative oxidase is repressed while cytochrome C reductase, ADP/ATP translocase and ATP synthase transcripts increased in abundance indicating a return to normal aerobic oxidative phosphorylation.

Analysis of 1000 nucleotides upstream of this alternative oxidase gene (AN2099.2) reveals two interesting putative transcription factor binding sites (http://www.cbrc.jp/research/db/TFSEARCH.html) and provides additional evidence for the metabolic role of this gene product. Beginning at position 968 from the putative translation start site is a Gcn4-like motif (score 97.4). In yeast Gcn4 is a general activator of genes involved in protein and purine biosynthesis (Hope et al., 1985). The Gcn4 homolog in *A. nidulans* is (CpcA) whose corresponding transcript is specifically upregulated under amino acid starvation conditions (Hoffman et al., 2001). Beginning at position 667 from the putative translation start site, an Ap-1-like motif (score 98.3) was found. In yeast, Ap-1 is a

transcriptional activator involved in response to oxidative stress, oxygen detoxification and metal resistance (Wu et al., 1993). Combined, these observations are consistent with the suggested functions of fungal alternative oxidases and expression of this gene by *A. nidulans* under the conditions tested. In the initial stages of growth (at 3 h) on pectin, the alternative oxidase gene and other genes may have been upregulated following transfer to fresh media as cells experienced general amino acid starvation conditions during acclimatization to pectin as a carbon source or the lack of a carbon source. One would expect the consequence of such induction to result in decreased ATP synthesis, yet allowing citric acid cycle turnover. However, this alternative oxidase was later repressed following acclimatization to pectin condition (but not occurring in glucose-starved condition) possibly to permit increased ATP synthesis and overall culture growth.

In conclusion, microarray expression data presented here provide useful fundamental results about the response of *A. nidulans* on a transcriptional level to a complex carbohydrate like pectin and under glucose-starved conditions at different times of growth. These results provide evidence of the manner in which *A. nidulans* modulates the expression of genes involved in carbon utilization and central metabolism.

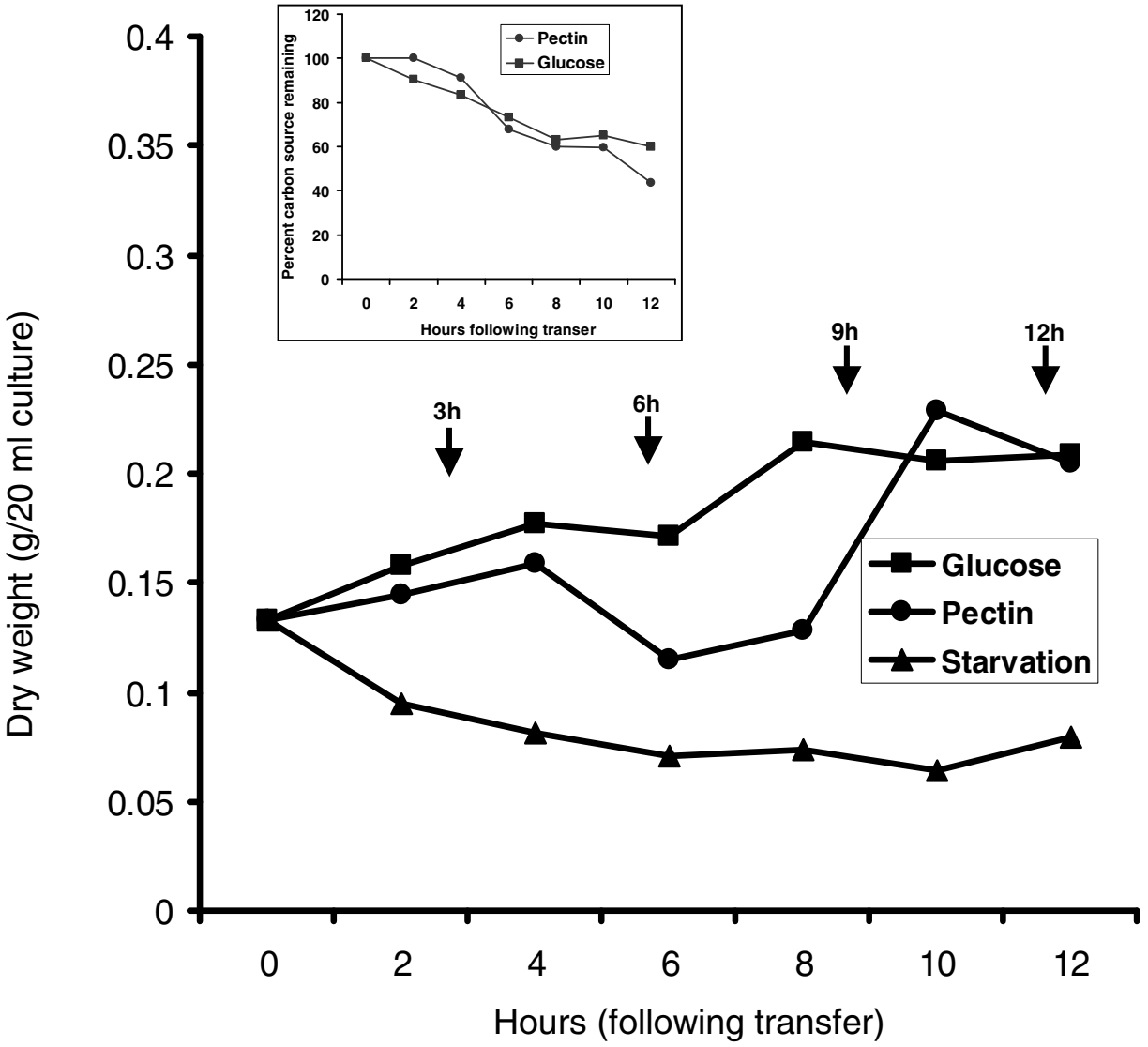

Figure 5. Growth curves of the fungus after the transfer from growth in glucose containing medium for 18 h to medium containing glucose, pectin, or no carbon source. The inset shows the degradation of pectin and disappearance of glucose from the medium during fungal growth. Fungal cultures incubated on pectin, glucose and no carbon source were collected at 2 h intervals and the dry weight of the mycelia was determined. The pectin remaining in the medium in which the fungus was growing was estimated by passing an

aliquot through a gel filtration column. The area of the void volume peak, representing relatively undegraded pectin, was integrated and compared to the peak obtained at 0 h (control). The concentration of the glucose in the glucose-grown medium was determined by labeling an aliquot of the medium with aminonapthalene tri-sulphonic acid (ANTS) and subjecting it to capillary electrophoresis. The black arrows at 3 h, 6 h, 9 h, and 12 h show the time points at which the mycelia were collected to perform the microarray experiments. The data are averages of three independent experiments.

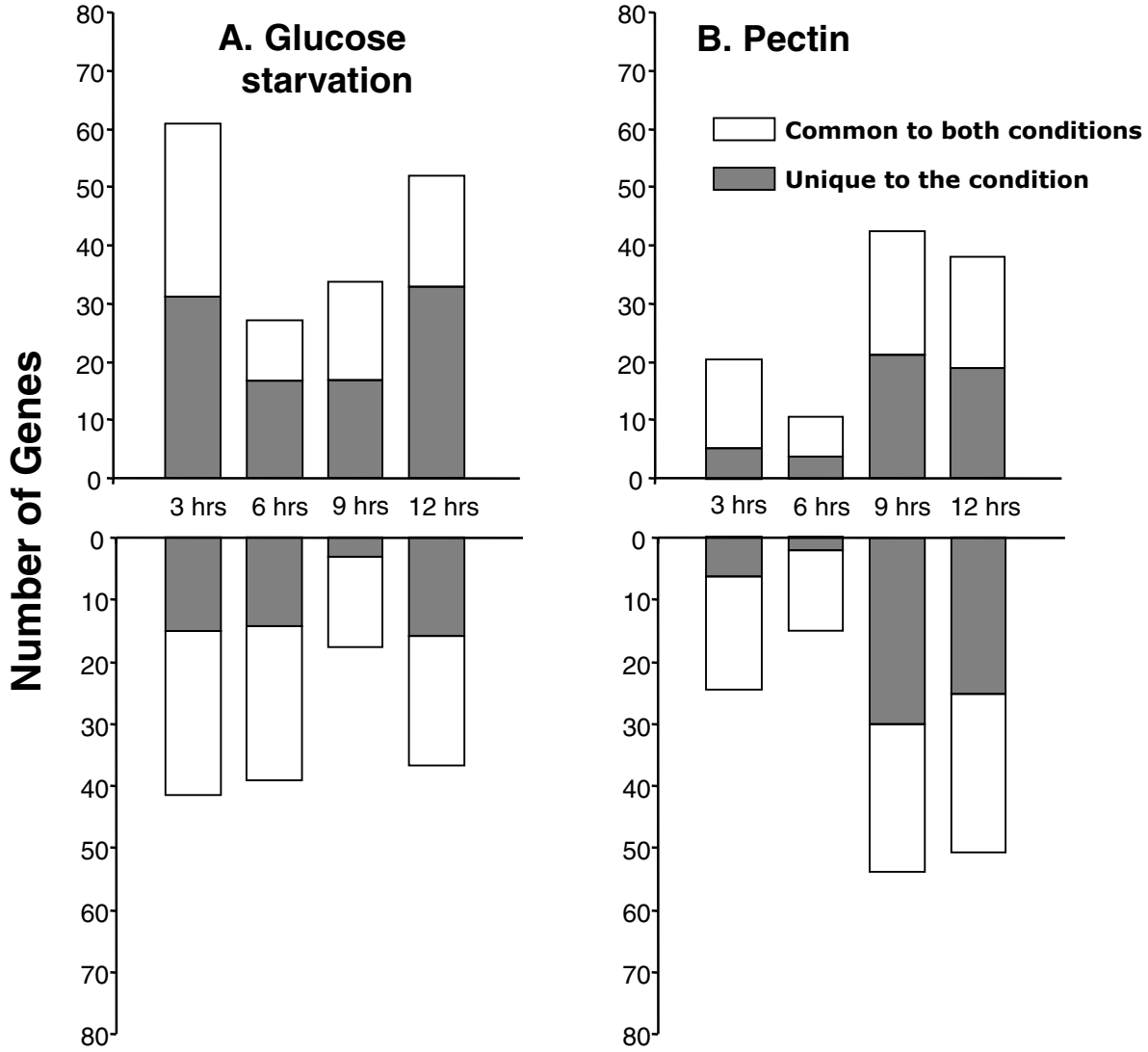

Figure 6. Distribution of pectin-regulated or starvation-regulated genes

DNA microarrays containing *A. nidulans* cDNA PCR products (1,184 unique genes) were used to assay gene expression in *A. nidulans* FGSC C26 following incubation in liquid media (**A**) devoid of a carbon source (glucose starved condition) or (**B**) containing 1% pectin. The bars reflect the number of genes that were induced or repressed at each time point tested. The gray segments reflect the number of genes that were specifically induced (upward) or repressed (downward) in either glucose starvation (panel A) or pectin (panel B) (i.e.,

condition specific regulation). The white segments reflect the number of genes, affected in at least one of the time points with the fungus growing on pectin and one of the time points with the fungus subjected to glucose starvation, that were induced (upward) or repressed (downward) at each time point (i.e., shared response).

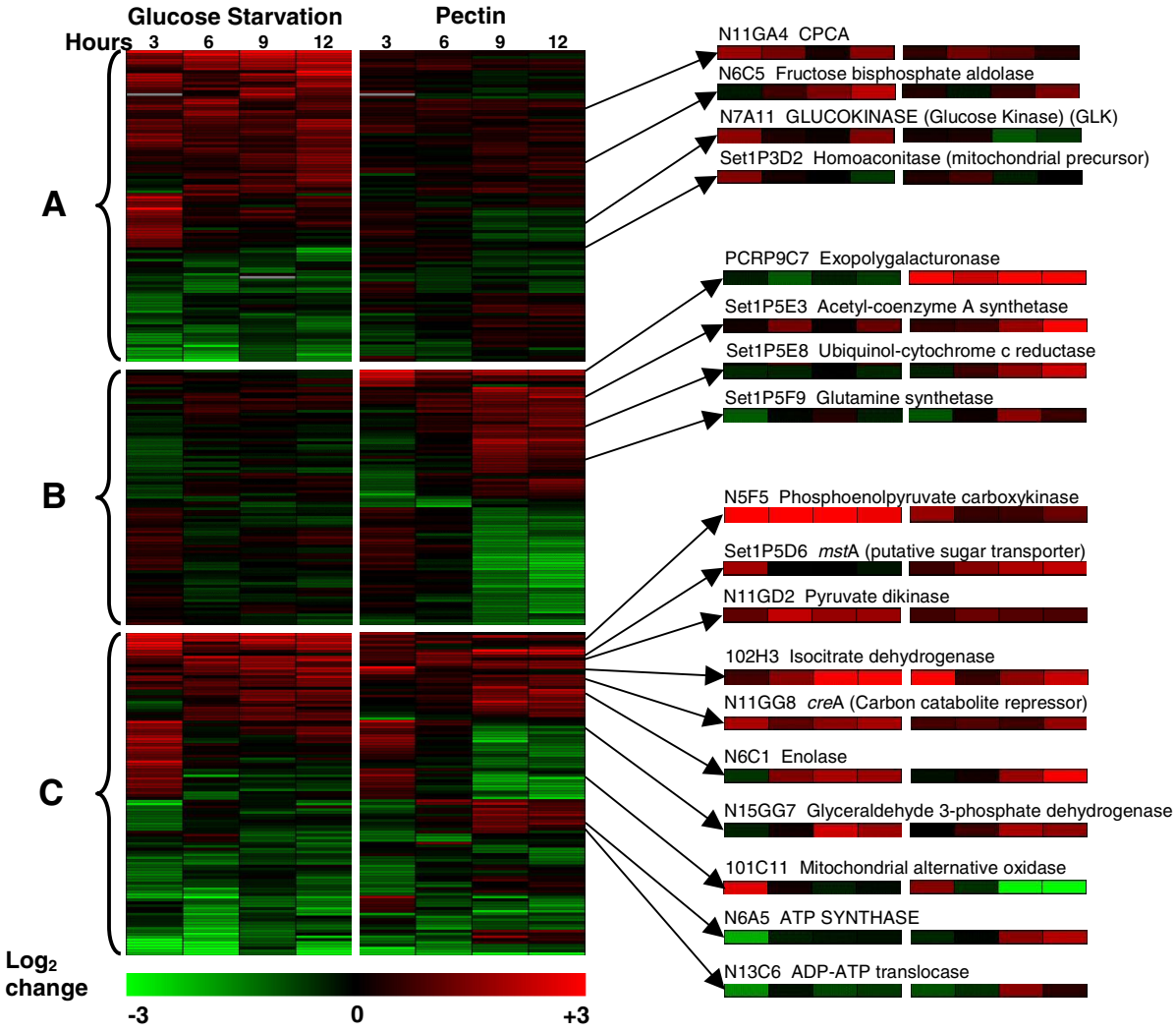

Figure 7. Hierarchical clustering of differentially expressed genes

The expression pattern of 312 genes is divided into three different panels depending on the observed responses. Panel A contains a total of 109 genes with a glucose starvation specific response, Panel B contains 89 genes with a pectin specific response, and Panel C contains 114 genes which showed a shared response. For each panel, the four time points of the glucose starvation growth condition are on the left while the four time points of the pectin growth condition are on the right. The expression patterns of select genes from each category is shown on the right-hand side of the diagram. Red indicates genes with increased transcript abundance and green indicates genes with decreased transcript abundance.

			Log$_2$ Ratios							
			Glucose Starvation				Pectin			
EST ID[a]	**An ORF[b]**	**Annotation**	**3h**	**6h**	**9h**	**12h**	**3h**	**6h**	**9h**	**12h**
Disaccharide/Polysaccharide metabolism, Sugar transport										
PCRP9C7	AN8891.2	Exopolygalacturonase	-0.230	-0.711	-0.258	-0.465	3.423	1.600	2.106	1.978
N13F9	AN2466.2	Glucose transporter	2.718	2.092	1.823	2.083	0.741	0.754	1.970	1.266
N7F12	AN6804.2	Related to myo-inositol transport protein	1.338	0.115	0.567	2.083	0.472	0.328	1.161	1.268
Set1P5D6	AN6669.2	MSTA protein	1.882	-0.041	0.005	-0.239	0.691	1.545	2.015	2.269
N13F9	AN2466.2	Glucose transporter	2.718	2.092	1.823	2.083	0.741	0.754	1.970	1.266
N12B2	AN2794.2	Hexose transporter	1.222	0.695	0.032	-0.088	-0.161	0.501	2.085	0.552
pncs919aG05	AN4148.2	Sugar transporter	0.453	-0.160	-0.922	-1.210	-1.213	0.531	0.942	-0.654
N11GF5	AN8400.2	Glucose transporter; TrHXT1	-0.398	0.899	0.236	-0.732	-0.769	1.091	1.359	1.269
pncs915aA3	AN2585.2	Monosaccharide transporter	0.451	0.312	-0.183	-0.390	0.560	0.162	-1.353	-1.415
MA96P1H12	AN3390.2	Pectinmethylesterase	0.509	0.355	0.208	1.038	0.706	0.211	0.553	0.227
Set1P3F8	AN6035.2	RTS beta	-0.018	0.174	-0.124	-0.378	2.301	0.769	1.935	2.092
Glycolysis/Gluconeogenesis/Pyruvate Metabolism										
N15GG7	AN8041.2	Glyceraldehyde 3-phosphate dehydrogenase	-0.283	0.133	1.633	1.221	0.009	0.473	1.310	1.124
N6C1	AN5746.2	Enolase	-0.410	0.993	1.306	1.195	-0.128	0.129	1.190	1.929
N5F5	AN1918.2	Phosphoenolpyruvate carboxykinase	2.237	2.316	2.485	1.856	1.176	0.421	0.466	0.807
N11GD2	AN5843.2	Pyruvate Dikinase	1.119	2.268	1.757	1.787	0.721	1.220	0.955	0.880
Set1P5E3	AN5626.2	Acetyl-coenzyme A synthetase	0.126	0.896	0.053	0.785	0.363	0.461	1.177	2.127
N6C5	AN2875.2	Fructose biphosphate aldolase	-0.187	0.456	0.957	1.477	0.247	-0.181	0.391	0.944
N8D1	AN1246.2	Phosphoglycerate kinase	-1.187	0.485	0.930	0.768	-0.118	0.324	0.938	0.584
N7A11	AN8689.2	Glucokinase	1.113	0.220	0.100	1.011	0.146	0.209	-0.676	-0.416
TCA/Glyoxylate										
102H3	AN2999.2	Isocitrate dehydrogenase	0.511	1.061	1.939	2.004	2.977	0.400	1.109	1.550
N12G5	AN2295.2	Succinyl-coa ligase	0.254	0.332	1.205	0.960	0.722	0.643	1.406	1.478
N12A4	AN2436.2	ATP-citrate synthase subunit 1	-1.021	-0.572	-0.078	0.187	-1.461	0.070	0.227	-0.315

N6H6	AN5634.2	Isocitrate Lyase	0.451	0.423	0.015	-0.660	-0.174	0.192	-0.471	-0.248
Set1P3D2	AN6521.2	Homoaconitase	1.023	0.147	0.008	-0.444	0.232	0.489	-0.244	-0.009
pncs919aF10	AN5571.2	Oxoglutarate dehydrogenase precursor	0.768	0.018	0.374	0.606	1.344	0.286	0.023	-0.043
N7E8	AN2916.2	Succinate dehydrogenase	0.310	-0.004	0.238	0.479	-0.030	0.039	0.088	0.016
N12G5	AN2295.2	Succinyl-coa ligase	0.254	0.332	1.205	0.960	0.722	0.643	1.406	1.478
Oxidative Phosphorylation										
pncs918aE07	AN9315.2	Apoptosis-inducing factor	1.299	-0.534	-0.345	-0.333	0.940	-0.312	-1.113	-0.148
101C11	AN2099.2	Mitochondrial alternative oxidase	1.764	0.142	-0.242	-0.097	1.044	-0.372	-1.933	-2.483
N13C6	AN4064.2	ADP-ATP translocase	-1.115	-0.146	-0.560	-0.448	-0.638	-0.375	1.068	0.331
N6A5	AN1523.2	ATP Synthase Alpha Chain	-1.388	-0.239	-0.174	-0.135	-0.302	-0.049	1.061	1.410
N5B9	AN2332.2	Succinate dehydrogenase	-0.024	0.405	-0.249	0.001	0.172	0.062	0.808	1.006
Set1P5E8	AN2306.2	Ubiquinol-cytochrome c reductase	-0.321	-0.294	-0.027	-0.311	-0.268	0.468	1.118	1.612
pncs920aB06	AN0357.2	Cytochrome c1, heme protein	-0.935	-0.632	-0.121	0.065	-0.546	-0.311	1.043	0.636
Lipid Metabolism										
pncs910aB11	AN6279.2	Carnitine acetyl transferase	1.787	-0.085	-0.399	0.804	1.397	-0.018	-1.677	-0.636
pncs917aD06	AN1037.2	Oleate delta-12 desaturase	-1.417	-1.360	-0.545	-1.130	-1.405	-0.304	0.659	0.330
Set1P5B11	AN0913.2	Cdp-diacylglycerol--inositol 3-phosphatidyltransferase	-1.978	-3.118	-1.097	-1.994	1.738	-0.411	-2.066	-0.792
N8E10	AN3662.2	Phytoceramidase, alkaline	0.500	1.621	1.244	0.606	0.360	0.421	0.341	-0.089
N5C5	AN6731.2	Stearic acid desaturase	-1.651	-1.699	-0.894	-1.326	-0.820	-0.641	-0.051	-0.226
N12A3	AN4135.2	Stearoyl-CoA desaturase	-1.925	-2.086	-0.825	-2.201	-0.837	-0.892	0.030	-0.206
N14GE4	AN8907.2	C-4 methyl sterol oxidase	-3.028	-3.619	-1.034	-2.151	-1.098	-1.755	0.582	0.224
N15GG10	AN4991.2	Aureobasidin-resistance protein	-0.687	0.308	-0.945	-2.060	-0.290	0.302	-0.581	-0.491
pncs919aB02	AN1176.2	Involved in sphingolipid biosynthesis	-1.065	-0.329	-0.242	-0.325	0.246	0.004	0.596	0.405
Set1P5H1	AN0054.2	Adenylate-forming enzyme	-0.630	-0.714	-0.288	-0.893	-0.891	-0.303	1.026	0.197
N7C1	AN2154.2	Phosphatidylethanolamine methyltransferase	0.321	0.470	0.450	0.663	0.209	0.297	0.585	0.542
pncs910aE06	AN3376.2	Squalene synthase	0.004	0.224	0.395	0.512	-0.219	0.096	0.688	0.508

Protein and Amino Acid Metabolism, Protein turnover

pncs916aF04	AN4376.2	Glutamate Dehydrogenase	-0.966	-2.074	-0.528	-0.725	-1.270	0.238	0.174	0.142
N11GC4	AN5134.2	Glutamate synthase	0.416	0.544	0.047	-0.083	0.545	0.868	1.261	1.565
N14GA7	AN4218.2	Elongation factor 1-alpha	-0.833	0.206	0.289	0.059	-0.914	-0.057	2.002	1.238
Set1P5F9	AN4159.2	Glutamine synthetase	-0.711	-0.055	0.274	-0.243	-0.655	0.103	1.070	0.433
N8G10	AN0856.2	Choline transport protein	-0.737	-0.665	0.227	-0.098	-0.082	-0.219	1.167	0.519
Set1P3B11	AN5783.2	20s proteasome component (beta 7)	-0.485	-0.077	0.028	0.237	-0.854	0.081	0.955	1.468
pncs918aF04	AN5055.2	Methionine aminopeptidase	-0.202	0.356	-0.050	0.551	-0.832	0.233	0.403	1.395
pncs917aD03	AN5444.2	Bifunctional tryptophan synthase TRPB	-0.051	-0.681	0.183	0.185	-0.713	-1.380	-0.618	-0.067
N5F11	AN0314.2	Aspartyl-tRNA synthetase	0.064	0.081	-0.258	-0.418	-0.565	-0.064	-1.052	-0.740
Set1P5H10	AN4869.2	Proteasome subunit	1.788	-0.227	0.969	1.255	1.565	0.146	-1.290	-1.155
N8D6	AN4794.2	40s ribosomal protein	0.490	0.301	0.853	1.497	0.108	0.001	0.329	0.364
N12H5	AN3359.2	Amino-acid permease	0.367	0.150	0.595	1.243	0.825	0.500	0.552	0.799
pncs910aC10	AN4925.2	NAAG-peptidase II	-0.297	0.009	0.661	1.054	-0.182	-0.149	0.597	0.242
N5H9	AN8881.2	Mitochondrial carrier AMCA	-1.287	-0.234	-0.365	-1.347	0.090	-0.018	0.406	-0.130

Regulation of Transcription

Set1P3C9	AN0083.2	Related to transcription initiation factor IIF 30K chain	1.131	0.528	-0.069	-0.239	0.462	-0.026	-0.560	-1.572
pncs918aB12	AN3179.2	Epa5p	1.310	-0.110	0.530	1.516	0.716	0.295	-1.723	-1.376
N11GG8	AN6195.2	DNA-binding protein creA (Carbon catabolite repressor)	1.343	0.712	1.093	1.301	0.518	0.625	0.466	1.143
pncs917aA02	AN3650.2	Binuclear zinc transcription factor	-0.817	0.385	0.454	1.104	-0.055	0.090	1.574	1.119
pncs916aB03	AN0388.2	Transcriptional activator xlnR	-0.129	-0.052	0.117	-0.119	-0.349	0.088	1.045	0.929
N11GA4	AN3675.2	CPCA	1.188	0.895	0.176	1.063	0.364	0.872	0.633	
pncs907aA02	AN3436.2	GATA-factor	1.739	0.456	0.357	1.047	0.287	0.283	0.323	0.614

Cytoskeleton, cytokinesis, cell cycle and signal transduction

N8C2	AN6523.2	Cytokinesis protein sepA	-0.010	-0.137	-0.072	-0.416	1.793	0.520	-0.846	-0.223
N12B11	AN8244.2	SNAD	-0.729	0.255	0.300	0.288	0.371	0.742	1.046	1.111
N12B12	AN8838.2	Tpr1 protein	-0.442	-0.053	-0.158	-0.260	0.106	0.367	1.088	0.597

Cell wall biogenesis										
pncs907aF12	AN5586.2	Mannose-1-phosphate guanylyltransferase	-1.571	-0.845	-0.427	-1.008	-0.811	-0.431	0.347	0.187
N6G7	AN8484.2	Cell wall galactomannoprotein	1.534	0.701	0.281	1.064	0.886	0.759	3.424	2.536
N11GB1	AN4390.2	Cell wall biogenesis protein	0.457	1.833	1.555	1.858	0.274	0.802	0.477	0.519
N15GA1	AN7950.2	Glucosidase	-0.856	-0.519	-0.807	-0.623	-0.362	0.024	-0.501	-0.839
N7G2	AN7657.2	GEL1 protein	-0.962	0.137	-0.194	0.165	-0.339	0.000	1.239	0.936

Table 1. Genes grouped into different metabolic pathways and other functional categories

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CHAPTER 4

DISCUSSION AND SIGNIFICANCE

The work described here helped us to isolate genes induced during growth in complex carbohydrates in the filamentous fungus, *Aspergillus nidulans*. The NSH technique helped us to obtain some of the genes and the microarray experiments helped us to characterize them. One of the problems faced by the *A. nidulans* community, inspite of the genome being completely sequenced, is the unavailability of a complete genome chip. Hence transcriptional profiling of *A. nidulans* using microarray experiments is not possible with the complete gene set.

We hoped to find many of the genes encoding cell wall degrading enzymes using the NSH method. However, we only found a rather small number of genes encoding cell wall degrading enzymes along with a large class of genes related to metabolism and genes with no known function. The cDNA library that we screened was constructed from fungal mycelia grown at pH 6.5 and many of the genes (xylanolytic and pectinolytic) encoding cell wall degrading enzymes are regulated by pH. Thus, it is likely that the growth conditions which we used would not have been conducive for the induction of all of the genes encoding cell wall degrading enzymes. The main factor involved in pH-dependent regulation in *Aspergillus* is the pH regulatory protein, PacC (de Vries et al., 2001). PacC activates alkaline-expressed genes at alkaline pH and represses genes activated at acidic pH. Under alkaline conditions, the C-terminal region of PacC undergoes proteolytic modification, which

helps it to bind to target regions (GCCARG with R being A or G) in the promoter regions of target genes (de Vries et al., 2001).

The results obtained from transcriptional profiling showed a shift in metabolism caused by the switch from glucose as carbon source to less readily metabolized polysaccharides. The effect of CreA binding on the promoter region of genes was investigated by analyzing the promoter region of genes upregulated in pectin only but not in glucose starvation and that group of genes upregulated in glucose starvation only but not in pectin. Our microarray studies indicate that mere derepression (i.e. removal of repressing carbon source) will not result in upregulation of all the genes. Similarly mere induction in the presence of an inducer will not result in upregulation of all the genes. Besides CreA, there could also be other regulatory factors and pathways playing a likely role in the upregulation of genes. Further studies with a *creA* mutant is essential to have a detailed idea about the gene expression mediated by carbon catabolite repression in *A. nidulans*.

In the past a wide variety of microorganisms has been screened for production of commercially useful polysaccharide degrading enzymes. The recent release of the complete genomic sequence of several fungi reveals that the redundancy of these enzymes is greater than was previously thought (http://afmb.cnrs-mrs.fr/CAZY/). Determination of the mode of action of each enzyme and a thorough analysis of the expression pattern of the various enzymes under a range of conditions in one convenient organism like *A. nidulans* should shed light on the biological rational of saprophytic growth on biomass.

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